

Will Europe be lost in space?

The European Space Agency has a good reputation and an even better prospectus, but it needs to be rid of the anachronistic doctrine of *juste retour*.

EUROPE'S space science community is learning by fits and starts the perils of its capital-expensive field — and that the perils are the same wherever people work in this capital-expensive field. Tales abound of how researchers have worked for years on the development of instruments to make observations from a satellite only to discover, late in the day, that there will be no funds to launch them on an Earth satellite or interplanetary probe. (The Britons who have been working with Italian groups on the development of a γ -ray telescope seem destined for that fate, see *Nature* 373, 459; 1995). Graduate students similarly work for years on such a project, but then run out of support before there are data to incorporate in a dissertation. And, generally, there are always fewer launches than the community considers it could use productively; the exploration of the Solar System is routinely squeezed by political imperatives.

These questions will be on the agenda for the ministerial meeting of the European Space Agency (ESA) planned for next November (and in the lobbying that will precede it), when ESA will be asking for member-states' support for its so-called 'Horizon 2000+' programme, entailing a 4–5 per cent annual increase of the budget in the first half of the next decade. Some of the forces that will then be deployed have already become plain. The British, for example, will be hoping to repeat in their dealings with ESA their success in containing the budget of CERN (the European particle physics laboratory at Geneva). The risk in that strategy is already plain. In space research, there seems still to be no shortage of governments willing to pay extra for a larger share of the action. At CERN last year, it was different; nobody wanted to pay more.

The British are on stronger ground in questioning the principle of '*juste retour*' by means of which member states hope for the return of a large proportion of their membership subscriptions by means of contracts between ESA and national industrial companies. This iniquitous principle goes back to the origins of ESA in the 1950s, when the agency (like its close cousin, the European Launcher Development Organization, now transmogrified into Ariane-space) was partly advertised as a means by which European companies could learn the techniques of satellite construction. Over the years, governments have become ever-more zealous in their expectations, thereby turning ESA into an instrument of European industrial policy, but a pointless one. But does every member state need a competence in satellite construction? The very notion fits awkwardly with

the reality of the European single market, in which business should flow to the most competitive companies.

But can member governments be weaned away from a practice to which they are apparently wedded? The obvious first need is for a survey of the space parts of Europe's aerospace industry, and for an appraisal of its future. On the face of things, there is no point in using the doctrine of *juste retour* if its purpose is simply to enable national governments to service ESA's modest construction needs up to some proportion of their annual subscription. Indeed, that is more like a guarantee that no European company will ever be competent enough to compete internationally with the major satellite constructors. Governments fond of saying publicly that most of what they contribute to ESA will be returned to national contractors should be asked whether, if ESA did not exist, they would pay over the same funds as straight subsidy. Mostly, they would demur, in which case they should pay their membership fees like grown-ups.

The cause is a good one. ESA's record is better than merely creditable, while the Horizon 2000+ programme, still in outline, is imaginative and potentially of great value. It is especially adventurous that the agency has the ambition to use the space environment as a physics laboratory and for astrometry and optical and infrared interferometry of objects in the Galaxy. The speaker at last week's meeting (see page 548) who declared the last project to be "one to die for" was merely saying that it is important to learn what the rest of the Galaxy is like. And that, of course, is what member governments should be paying for, not for a mechanism of industrial policy for which Europe has no urgent need. □

Clinton's dark horses

The putative Surgeon-General of the United States should command his president's support.

THE post of Surgeon-General in the United States is not usually contentious, although holders of the post can get themselves into trouble if they put their minds to it. In the 1960s, a surgeon-general famously caused embarrassment by announcing a ban on phosphorylated detergents (advertised by environmentalists as a threat to civilization as we know it through eutrophication) and their substitution by chemicals afterwards shown to be carcinogenic. More



recently, Dr Everett Koop (President George Bush's Surgeon-General) irritated his political masters by speaking out on AIDS, and on the means by which people might avoid it. President Bill Clinton has been no luckier. That, at least, is what he may think.

At the end of last year, Clinton demanded the resignation of Dr Joycelyn Elders after she had told a meeting of a UN committee on sex education that children should be told something about masturbation. Her opinions were widely and even sensationally reported. There is no suggestion that she was advocating onanism as a male way of life, or even as a means of contraception, but merely that she regarded this information about the working of the human body as necessary to young people's understanding of themselves. But the opinion was evidently judged politically incorrect. Humbugged indignation was everywhere to be heard. The president did not lift a finger to defend Elders. She was quickly gone.

Clinton is no luckier, as he will see it, with the project to replace Elders with Dr Henry Foster, a black gynaecologist from Tennessee whose standing with his professional colleagues in the United States is unquestioned. The trouble is that Foster, being a gynaecologist, has in his time performed abortions on pregnant women. Saving those who have exercised the proper conscientious right to decline, what practising gynaecologists has not performed a few operations of this kind? But with two (male) 'abortionists' already murdered on that account in the United States, and with the anti-abortion lobby more sure than ever that it has Congress on its side, it was inevitable that a gynaecologist nominee for the post of surgeon-general would be given a drubbing in the Senate's confirmation hearings.

That is how it has been, but worse. There are differing accounts of how many abortions Foster has carried out or supervised. The White House says "fewer than a dozen", Foster himself acknowledges "39", but others claim that the number is much larger. Nobody suggests that, whatever the number, Foster has behaved illegally, nor would it be entirely unreasonable that a busy gynaecologist should have carried out some hundreds of abortions in a professional lifetime. The plain truth is that the number is entirely irrelevant to Foster's competence for the post to which he has been nominated. Nor should it matter that he may have used hysterectomy as a means of sterilization more than 20 years ago. It is also plain that the White House appears not to have had the wit to anticipate the sensitivity of this issue. Is it possible that the people there are ignorant of what gynaecologists do for a living, and too busy to find out?

In the past two years, Clinton has made several good appointments and as many bad ones. He has a reputation for letting his nominees swing in the wind when the Senate committees make the going rough. On this occasion, he has a duty not merely to give Foster his full backing, but to insist that the number of legal abortions a gynaecologist may have carried out is strictly irrelevant to his competence as surgeon-general. Unless he fights for Foster, the Senate may deny him all the appointments he wishes to make in the two years ahead. □

France's blood scandal

France should commission an independent inquiry in the contaminated-blood saga.

THE mob's thirst for vengeance can take precedence over justice. Many of the darker decisions of the tribunals in the French Revolution showed that, memorably in the case of Lavoisier. So did a Paris tribunal last week, when it refused parole to one of the four men convicted over the contaminated-blood affair, on the astonishing grounds that his release would prevent "appeasement of the justifiable resentment of the victims" (see page 550). The ruling lends credence to the cynical view that the French judicial system, under pressure from the public, has not let facts or the law impede its search for scapegoats. As well as the four people already tried and convicted, those now arraigned include Laurent Fabius, a former prime minister, and François Gros, the distinguished cell biologist and former government adviser.

Circumstantial evidence supports the suspicion that the courts all along have delivered the verdicts that the public wanted. Despite the complexity of the case, the court that heard the initial trials did not commission an independent expert report. The investigation of the main charge (that haemophiliacs were given HIV-contaminated clotting factors when heat-inactivated alternatives were available) was carried out rapidly by judges and police, and was full of errors. Thus a mistranslation of a text from October 1984 led the rapporteur to the Conseil d'Etat — France's highest court — to affirm that, on that date, the efficacy of heat-inactivated blood products "should be taken as established"; the original text reads "remains to be proven". Similarly, the rapporteur claimed that Luc Montagnier, from the Institut Pasteur in Paris, stated at a meeting on 8 October 1984 that the "transmission of the virus by Factor VIII was possible but that it was inactivated by heating". Montagnier had actually referred to a solution of the virus in a test tube, which is a very different matter.

The danger now, for France, is that the hunt for scapegoats will become perpetual. Who is to say, and how, when the "appeasement of the justifiable resentment of the victims" will be complete? The French press, which has played the part of the women who used to take their knitting to the guillotine to urge on the executioners, is unlikely to be much help. The victims themselves, or the relatives of the real victims, who must sense they are pushing at an open door, are unlikely to declare themselves content. Yet at no point in the past trials have the courts taken sufficient account of the many extenuating circumstances of the mid-1980s — uncertainties about the importance of AIDS and the quality of reagents on the market. So what will happen if Fabius and Gros are sent to jail and appeasement remains elusive? Better act now to avoid that evil prospect. The government of France needs to get off the unjust road it has been travelling, and may find that a serious inquiry by an international commission is the only way of doing so. □

US spent-fuel ban leaves European research reactors out in the cold

Munich. A federal district court in South Carolina has confirmed an earlier ruling that the US Department of Energy (DoE) cannot require the state to accept spent fuel rods from European research reactors intended to be held in storage at the DoE's nuclear site at Savannah River.

As a result, the United States may have to renege on a previous promise to accept such spent fuel, leaving many European research reactors in crisis as their own temporary storage facilities are no longer able to meet their needs.

As part of a broad campaign to limit the spread of nuclear weapons, the United States agreed in 1978 that it would take back spent fuel rods of US origin from foreign research reactors if the countries concerned agreed to use low-enriched uranium (LEU) instead of bomb-grade high-enriched uranium (HEU). This is known as the Reduced Enrichment for Research and Test Reactors (RERTR) agreement.

Nearly all those concerned agreed to make the change, despite the considerable expense involved. But in 1988, pressure from anti-nuclear groups led the United States to accepting spent fuel. Then in 1993 the Clinton administration announced a renewed commitment to anti-proliferation, requiring in particular a full environmental impact statement on the consequences of accepting foreign spent fuel.

Reactivating the agreement with foreign research reactors, however, has proved difficult. The return of spent fuel has been

criticized on environmental grounds, and critics also point out that, with reprocessing stopped at Savannah River, there is no economic advantage in using interim storage.

There are now estimated to be 15,000 spent fuel rods in non-US research reactors awaiting a firm US decision. For some reactors, the issue is critical. Following an inspection of foreign facilities last summer, the DoE agreed to take back and store 409 rods from eight European reactors whose own storage facilities were already full.

The first shipment, comprising 153 rods from four European research reactors, was despatched last September. But an injunction by the South Carolina federal district court prevented the shipment from landing, the state arguing that the risks to the state's population could not be assessed until a full environmental impact statement was complete (see *Nature* 371, 192; 1994).

The injunction was lifted some days later when the court accepted arguments by the DoE and the Nuclear Control Institute (NCI), an independent watchdog group, and various environmental groups that the immediate risk of terrorist attack on the shipment outweighed the likely risk to the local environment.

But the court also demanded an immediate full hearing. This was completed last November, and on 26 January the judge ruled that an import ban on the remaining 256 fuel rods would be maintained, at least until the environmental impact statement is published later this year.

An appeal by the DoE is likely to be heard within a few weeks. The department wants the ban overturned as soon as possible so that a second shipment, carrying spent fuel from three research reactors in Germany, Greece and Switzerland, and scheduled for this spring, can go ahead.

Thomas Robertson of the Hahn Meitner Institute in Berlin, where the German reactor is located, says that if the United States is unable to find a solution, it could fall back on a second option, namely an agreement signed in 1990 with the reprocessing facility at Dounreay in Scotland.

But Robertson says that the institute would prefer to return the fuel to the United States, as the Dounreay plant handles only HEU, so reactors would have to abandon their plans to convert to LEU.

In contrast, Panagiatis Manakos, chairman of the Democritos research centre that houses Greece's research reactor, says that Greece has no alternative available. "We hope, and are optimistic, that the United States will honour its long-standing commitments, which have been the basis of our planning for our reactor," he says. The Greek reactor is in the process of converting to the burning of LEU.

There have been reports in the United States that some DoE officials have considered making a deal with South Carolina to persuade the state to accept the fuel rods — for example, by looking at the possibility of re-opening the reprocessing plant at Savannah River. But this has been denied by Hazel O'Leary, the energy secretary.

Some anti-proliferation campaigners are using the recent US decision to stop construction of the proposed Advanced Neutron Source (ANS) — a research facility which was to have burnt HEU — to argue against a new research reactor, the FRMII, which the Technical University of Munich plans to build at Garching, in Bavaria.

But Klaus Böning, the director of the FRMII project, says that the ANS decision was based on cost, and was not related to concern about nuclear proliferation. It has "no consequences" for FRMII, he says, which, despite vehement local opposition, has already secured funding agreements from the state of Bavaria and the federal government.

The opposition Social Democrat Party (SPD) has, however, called for the FRMII to be reassessed after the US decision. The SPD's parliamentary group has called on the federal government to reopen negotiations with Bavaria and the Technical University of Munich about halting the programme.

Alison Abbott

Norway honours space physics pioneer

Oslo. The government of Norway has honoured Kristian Birkeland, one of the world's earliest space physicists, by printing his portrait on a new 200 kroner (US\$30) banknote (right).

Birkeland, who was born in 1867, began his academic career as a mathematician, and was the first physicist to complete a general solution of Maxwell's equations. He is perhaps best known as a researcher for identifying the mechanisms behind the aurora borealis — as well as for his efforts in establishing a network of observation stations in the Arctic.



But he also had a strong practical bent, registering 59 separate patents that included the plasma arc that led to the first industrial process for nitrogen fixation. This discovery formed the basis of what has become one of Norway's largest companies, Norsk Hydro, and provided Birkeland with an income to finance his auroral research. □

ESA eyes the attractions of small space missions

London. The European Space Agency (ESA) is considering the possibility of launching a series of relatively small space missions as one of a number of steps that might increase the cost-effectiveness of its space science programmes.

Speaking at a meeting in London last week organized by the UK Particle Physics and Astronomy Research Council (PPARC), Roger Bonnet, the director of the ESA programme, said that a study is also being made of the relative costs of ESA's administration of space projects compared to similar costs in the private sector.

He added that the agency was also looking at the costs of using the controversial *juste retour* principle in financing space missions, under which the 14 countries that contribute to ESA's budget have agreed that contracts worth at least 96 per cent of each country's subscription should be awarded to its national aerospace companies.

But Bonnet refused to make any concessions to ease the plight of British scientists who have been refused funds by PPARC to provide the payloads for the Integral gamma-ray satellite, even though this mission was initially suggested by astronomers from the United Kingdom (see *Nature* 373, 459; 1994).

ESA's existing and planned space missions fall into two categories: the so-called cornerstone missions, four of which form the foundation of its science programme Horizon 2000, and a series of medium-sized missions. The former cost a maximum of ECU600 million (US\$750 million) each, and the latter ECU345 million.

While acknowledging that large amounts of money are needed for major projects such as planetary missions, some scientists are concerned that they inevitably lock researchers into related fields of study — reducing prospects for the pursuit of other scientific goals. Another concern is that budget constraints restrict the frequency of large and medium missions.

One suggestion at last week's meeting to compensate for both factors was that the European agency should consider launching a series of small missions costing around ECU50million, perhaps launching one every two years over a 20-year period.

As an example of a successful 'small mission' — even if its price was slightly higher — Leonard Culhane, director of the Mullard Space Science Laboratory, pointed out that the 380-kg Yohkoh satellite launched by Japan in 1991 with a British instrument had "revolutionized" studies of the physics of the Sun (see figure). "I think that ESA would be wise to move in this direction," Culhane said.

In response, Bonnet pointed out that

although ESA had been given a mandate to explore small missions three years ago, there had been little progress in defining what a small mission really is. "There is often a confusion between 'small' and 'cheap'," he said. "Furthermore, some seem to believe that a 'small' mission is merely a way of making a 'medium' mission cheaper."

Nevertheless, Bonnet said that the idea, which is already being studied by other agencies (including the US National Aeronautics and Space Administration), is now the subject of two separate studies within ESA, one a general assessment of the implications of such missions, the second a more detailed study of possible candidates.

A workshop on small missions, involving scientists, industrial representatives and members of ESA's science programme com-

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Small is beautiful: a picture of the Sun taken by the 380-kg UK/Japan Yohkoh satellite.

mittee, is due to be held in the spring, with its conclusions being forwarded to the ESA council. Firm proposals are expected to be put to the ministerial meeting in October.

That meeting will also discuss the other moves Bonnet is making to increase the cost-effectiveness of ESA's space missions. The British government, for one, is keen to reassess the *juste retour* principle, still smarting at having lost the contract last year to build the X-ray satellite XMM to a German contractor because of this principle, despite having produced the most highly-rated bid (see *Nature* 371, 545; 1994).

But some British space scientists are worried that the United Kingdom may be painting itself into a corner, and will find itself isolated at the October meeting. For example, it appears that other countries may be prepared to pick up the contracts for the Integral payload dropped by PPARC. (In addition to Italy's extra support for the imaging telescope, Spain is said to be prepared to provide new funds for the optical transient camera.)

David Dickson

Budget cuts will put Russian agency in 'critical' condition

Moscow. As the US National Aeronautics and Space Administration celebrates last week's successful rendezvous with the Russian Mir space station, the director of the Russian Space Agency, Yuri Koptev, has said that financial problems facing the agency could lead it to curtail significantly the future operation of the space station.

Also under threat, said Koptev, are his agency's plans to launch 11 manned space flights. The agency's financial difficulties are the result of broad cuts on spending on research which are leaving the Russian space programme in a "critical" condition, he said.

The wider difficulties facing Russian science have been acknowledged by Boris Saltykov, the minister of science and technology policy, who criticized the lack of support from his government colleagues at a meeting of the State Duma (parliament)'s committee on education, culture and science.

According to Saltykov, science is now the lowest priority of 18 separate items in the national budget, and last year the scientific community received only 52 per cent of the funds allocated to it by the Duma. "All national leaders claim that science has priority in their policies," he said. "They should either openly acknowledge that they have changed their minds, or agree that their deeds do not match their words."

One result of the cuts is that state-owned scientific centres alone now face unpaid bills totalling 250 billion rubles (about US\$60 million). "We have been offered discounts on our electricity bills, but local authorities are reluctant to accept this decision as they have not received any special funds to cover the costs," said Saltykov.

Questioned about his decision to endorse the 1995 budget, Saltykov said he had been faced with two alternatives: to accept a budget promising large amounts of money for science, less than half of which was likely to materialize, or to accept a more modest budget with a high chance of it being met.

"Naturally we preferred the latter," he said. "Last year we were promised 5.1 thousand billion rubles, but only received 2.9 thousand billion rubles. This year's budget allocates 5.5 thousand billion rubles for science; if we really receive the money, the budget will have been doubled."

Yuri Osipov, the president of the Russian Academy of Sciences, said that although government spending will increase the academy's budget by 10 per cent next year, this is only half of what is needed.

Meanwhile, in an interview with the news agency Interfax, Koptev said that the draft 1995 budget allocated only 1,200 billion rubles for space exploration — about 40 per cent of the 1994 figure.

Carl Levitin

Panel to propose shake-up for NASA labs

Washington. A panel set up to review the 12 research centres run by the US National Aeronautics and Space Administration (NASA) has concluded that some of the work should be consolidated to avoid duplication, and that other work should be farmed out to industry and universities.

But a draft report describing the panel's recommendations stops short of calling for any of NASA's research centres to be closed, and dodges the question of which functions should be cut at which locations.

These shortcomings drew criticism last week from NASA's external advisory council, which was briefed on the draft report by the panel's chairman, John Foster, a former head of research at the Department of Defense. As a result, the panel may recommend specific cuts when it issues its final report in early March.

The Foster panel, part of a review of federal laboratories which is scheduled to be completed in April, is NASA's equivalent to the Galvin commission that recently reported on the Department of Energy's laboratories (see *Nature* 373, 463; 1995). A similar exercise is being carried out at the Department of Defense.

NASA's centres range from research-orientated laboratories such as the Ames Research Center near San Francisco and the Goddard Space Flight Center in Maryland, to the Johnson and Kennedy Space Centers, which are more focused on human space-flight. Since NASA was founded in 1958, each centre has developed capabilities in a wide range of disciplines — and Foster says that is part of the problem.

"Almost every centre is into almost everything," he told the advisory council at a meeting in Washington last week. While some overlap is good, Foster said, much of it

is unnecessary. In an era of high-speed communications, for example, he questioned the need for each laboratory to have its own state-of-the-art computer facilities and fleet of aircraft.

Rather than competing as independent fiefdoms, said Foster, the centres "have to become dependent on one another". Achieving that, he said, "means shutting down some of the capabilities in many of the centers".

The panel also recommends that the space agency should transfer to industrial and university contractors some research now carried out in-house at the centres. Some centres might even be turned over entirely to universities says Foster, in much the same way that the California Institute of Technology in Pasadena operates the Jet Propulsion Laboratory.

But the "quick and dirty" study, which only began last October, contains few specific proposals. Panel members who briefed the advisory council last week admitted they had had neither the time nor the expertise to examine in detail which programmes could be cut or transferred out of the centres.

Ironically, NASA's own cost-cutting exercises over the coming months are likely to be much harsher. Even before Congress starts whittling away at its budget, the space agency is already facing serious financial problems later in the decade.

The White House's Office of Management and Budget (OMB) informed NASA managers in mid-January that the agency's budget will drop from \$14.3 billion in 1996 to \$13.1 billion in 2000. Much of this reduction is to pay for President Bill Clinton's proposed middle-class tax cut.

The administration has further specified that the reductions should not come out of

the space station's \$2.1 billion annual budget. Daniel Goldin, the administrator of NASA, has imposed additional constraints by safeguarding new projects, particularly research into a new Reusable Launch Vehicle (see *Nature* 373, 180; 1995). "If we have budget problems, we are not going to cut this programme," Goldin told the advisory council.

That challenge has sent NASA's money managers into something of a panic. "We have no idea how we're going to take [the cut]," Malcolm Peterson, the agency's deputy comptroller, told the advisory council. Goldin's solution, which he says is going to be "very painful", is to cut back on people and infrastructure, not programmes.

The agency plans to halve its Washington headquarters staff, and may slash its total work force, currently more than 22,000, by as much as a third. Shutting down whole centres would be the most drastic option. But it would also create political problems with congressmen affected by the loss of jobs in their states.

Tony Reichhardt

Merck releases first 'gene index' sequences

London. Merck & Co., the US-based pharmaceutical company, announced last week that, under a project launched five months ago with researchers from Washington University in St Louis, Missouri, it is making publicly available the first 15,000 expressed human gene sequences that have resulted from a collaborative effort to identify cDNA clones for expressed human genes.

The goal of the project, known as the Merck Gene Index, is to process about 300,000 human gene sequences over the next 18 months, with sequence data being submitted to the Expressed Sequence Tag (EST) division of Genbank for immediate distribution at an anticipated rate of 4,000 sequences a week.

The decision to make the gene sequences freely available to researchers was said at the time to be a response to a decision by Human Genome Sciences, following a \$125-million agreement with Merck's rival SmithKline Beecham, to impose conditions on the terms under which scientists would be allowed access to sequences obtained by its Institute of Genomic Research (TIGR) (see *Nature* 365, 371; 1994).

Meanwhile TIGR has announced that, working in collaboration with various chromosome-mapping laboratories in both the United States and France, it intends to construct an expression map of the human genome, pinpointing the location of individual genes. TIGR says that the mapping will start with 40,000 ESTs that are already publicly available. □

Alison Abbott

Italy retains existing *concorso* rules

Munich. In his first public statement as Italy's new minister for research and universities, Giorgio Salvini last week announced his decision to call a new *concorso* — the public competition for full-time university academic positions — claiming that universities can no longer afford to wait for agreement on new rules.

Salvini also announced his support for a three-year plan for research and development in Italy, which is now before parliament. The plan would hold the budgets for most research agencies at current levels. But it cuts funding for the National Agency for New Technology, Energy and the Environment (see *Nature* 372, 393; 1994).

There has been widespread demand in Italy for many years for reform of the system of academic promotion because it lacks transparency and formal criteria for deci-

sion-making and it is therefore seen as being vulnerable to abuse.

Umberto Colombo, research minister until last summer, initiated a reform of the system, and his successor Stefano Podestà drafted a bill with this objective. But although some of the proposals were widely accepted, such as the requirement that all candidates for tenure should have a PhD, other proposals proved more controversial.

For example, there was considerable opposition to a proposal in the bill that long-serving associate professors could become full professors simply with the agreement of a local university committee.

Salvini has now decided to start immediately a new series of *concorsi* for full and associate professorships according to the existing rules. The last full *concorso* took place in 1989.

British MPs 'likely to oppose gene patents'

London. As the European Parliament prepares to vote on whether human genes should be patented, members of an all-party committee of Britain's House of Commons indicated last week that they are likely to recommend that patents should not be allowed on "naturally occurring nucleotide sequences".

Alan Williams (Labour, Carmarthen), a member of the Commons' Select Committee on Science and Technology, which is conducting an inquiry into human genetics, said there is a "growing consensus" among committee members that DNA sequences should be considered as "pure knowledge" — and thus should not be patented.

Williams' European colleagues are due to vote on 1 March on harmonizing biotechnology patent legislation throughout the 15 member states of the European Union. In particular, they are being asked to endorse a controversial draft directive arrived at after seven years of negotiation between the European Commission, the Council of Ministers (representing EU states), and the European Parliament (see *Nature* 372, 310; 1994).

A compromise text, agreed by representatives of each of the three organizations last month in a so-called "conciliation committee", would allow patents on human genes. These would be patentable as parts of the human body that are "obtained by a technical procedure in such a way that they cannot be linked to a specific individual".

Despite the agreement on a common wording, separate interpretations of the draft have been put forward by the council and the parliament. The council emphasizes that the proposed directive would allow for parts of the body (such as genes) to be patented once they have been isolated from the 'human environment'.

In contrast, parliamentarians point out that they have successfully protected the 'genetic identity' of individuals, for example for disallowing patents on a DNA sequence that can be traced to a single individual.

Speaking after the conciliation meeting, Willy Rothley (PSE, Germany), one of the strongest critics of the commission's earlier position on the patenting of genes, said that the new text represents a "considerable improvement" on the draft originally put forward by the commission.

Rothley emphasized the parliamentary delegation's success in tempering purely commercial interests in patents. "The European Parliament has been able to impose an ethical dimension on patent rights and has been able to obtain most of the guarantees that it was asking for," he said.

But it remains to be seen whether his assurances are sufficient to persuade the European Parliament to support the compromise position. To achieve this, the draft directive will now have to be approved by a simple majority of parliamentarians attending a debate on the directive.

Supporters will include those who feel that, even though the new text does not go as far as the parliament had previously sought, they have achieved recognition that the concept of 'human dignity' should be included in patent legislation. But the compromise will be strongly opposed by the Greens, who are demanding much harsher restrictions on patents — in particular, that they should not be granted on either human genes or on transgenic animals.

"We feel that Parliament, having voted previously against patents on parts of the human body — including genes — under any circumstances, is morally obliged to reject this compromise", says Linda Bullard, a staff member of the Green Party. "This is

not a question of individual human dignity, but collective human dignity."

British parliamentarians who expressed opposition to patents on DNA sequences (including genes) during a hearing in London last week adopted a more pragmatic approach, arguing that giving companies the rights to nucleotide sequences could interfere with the ability of researchers to pursue complex genetic diseases.

The current system was defended by Paul Hartnack, the comptroller-general (head) of the UK Patent Office, who claimed that it is sufficient to disallow patents on gene



sequences of unknown function. Once a gene has been discovered and its function identified, he claimed, the work stemming from this discovery is basically "applied research" — and so would be adequately covered by existing licensing practices.

Members of the committee, however, argued that this did not address the full complexities faced by research teams working on diseases caused by the interaction of several genes, where uncertainty about the relative licensing rights of the 'owners' of each gene could put off potential sponsors of the research.

"Our concern is that if someone discovers and then patents a particular gene, that will increasingly complicate, slow down and make more expensive the process of working out its interaction with other genes in the human body," said committee member Spencer Batiste (Conservative, Elmet).

Derek Wood, the Patent Office's chief examiner for biotechnology patents, received a sceptical response from the committee when he claimed that a gene isolated from the genome is not the same as when it is found in nature on the grounds that the former has had its introns deleted.

The select committee's report, which will cover all aspects of human genetics, is not due to be published for several months. But the scepticism expressed by its members at last week's meeting may well reverberate during the European Parliament's debate next month.

David Dickson

Blood transfusion chief is refused parole

Paris. A Paris tribunal last week reversed the decision of a parole board to release Michel Garretta, the former head of the French National Blood Transfusion Centre (CNTS), who was imprisoned in 1992 for supplying haemophiliacs in the mid-1980s with blood products contaminated with HIV (see *Nature* 364, 269; 1993).

The tribunal was convened following an appeal against the parole board's decision by the public prosecutor's office. That appeal — an unusual step in France, as parole board decisions are rarely contested — followed a demand from the minister of justice, Pierre Méhaignerie, that Garretta should remain in prison because of the need to maintain "public order".

The tribunal subsequently decided that Garretta's release would interfere with the "appeasement of the justifiable resentment

of the victims". The decision sent shock waves through the legal establishment, as it established that public opinion can play a determining role in parole cases.

The tribunal's ruling comes six months after the Paris bar association publicly warned the legal system against bowing to public pressure in the contaminated blood affair. Writing recently in the journal *Médecine et Sciences*, Axel Kahn, of the Cochin Institute of Molecular Genetics in Paris, compared the affair with previous instances in French history when individual men have been wrongly convicted to satisfy public opinion.

The newspaper *Le Monde* alluded to the same risk in an article about last week's ruling, stating that "if the idea of vengeance enters the courtroom, we renounce the state of law".

Declan Butler

Japanese bureaucracy reforms may only pierce skin deep

Tokyo. The Japanese government's much vaunted plans for a radical reform of its semi-government organizations (*tokushu hojin*), some of which run much of the government's research and development activities, has turned into a feeble reorganization of only a handful of such bodies.

At present, there are 92 *tokushu hojin* under various ministries and agencies, responsible for spending more than ¥4,000 billion (US\$40 billion) a year in taxpayers' money. Their proposed reform is a key policy of the administration of Prime Minister Tomiichi Murayama.

The reform is also controversial. Only a few weeks ago, a senior official at the Science and Technology Agency (STA) lost his job after resisting calls for reform by Makiko Tanaka, the cabinet member who heads the agency (see *Nature* 373, 177; 1995). But now that the plans have been released, it is difficult to see what all the fuss was about.

The STA oversees six *tokushu hojin*. These include several large research and development organizations, such as the Institute of Physical and Chemical Research, the National Space Development Agency, the Power Reactor and Nuclear Fuel Corporation and the Japan Atomic Energy Research Institute.

But these will be left untouched. The only change for the STA will be the 'merger' of two much smaller organizations, the Research Development Corporation of Japan (JRDC) and the Japan Information Center of Science and Technology (JICST).

There is no overlap between the activities of these two bodies. JICST provides (and in some cases sells) information services on Japanese science and technology, and maintains part of the government's computer network system linking research organizations in Japan and overseas.

JRDC supports the development of technology in the government sector and its transfer to industry. It is perhaps best known for its ERATO (Exploratory Research for Advanced Technology) programme which provides large grants to joint teams of young researchers from government institutes, universities and industry.

JRDC and JICST officials are bemused by the merger. A JRDC official says that until December they were under the strong impression that JICST would be privatized, had been discussed for many years.

JRDC was until recently in the same building as JICST in central Tokyo. But it moved last year to Kawaguchi city in the northwest suburbs of Tokyo as part of a government policy of decentralization. JICST, on the other hand, was about to move to another office in central Tokyo as part of its (now cancelled) privatization.

According to one JRDC official, fax messages are "flying back and forth" between JICST, JRDC and STA to establish what the merger means. But he says that his organization has been assured by the STA that none of its programmes will be affected. Indeed, past experience suggests that the merger will merely result in a reshuffling of staff, with few (if any) redundancies.

An official at one of the organizations says that other larger *tokushu hojin* under STA may have been left untouched — despite Tanaka's call for reform — because Murayama, a socialist, wanted to avoid the labour disputes that would inevitably be involved in a major reorganization. He says the combination of Murayama and Tanaka, an outspoken member of the right-wing Liberal Democratic Party, has caused "great confusion" in the agency's reform plans.

Changes are also relatively minor in other science-related ministries. The New Energy and Industrial Technology Organization (NEDO), which administers most of the research and development projects of the Ministry of International Trade and Industry (MITI), will be merged with MITI's Coal Mining Areas Restoration Agency.

But NEDO already has a section that deals with the problems of land subsidence caused by collapse of old coal mine shafts. The agency will therefore be absorbed into NEDO without any disruption of NEDO's prime purpose, namely funding research and development in new technologies.

Indeed, most of the reform plan consists of such mergers. In total, 14 *tokushu hojin* will be merged into seven. Only one body — the Institute of Social Security Research — will be abolished. And even that will not really disappear, as it is to be incorporated into a reorganization of seven national research institutes under the Ministry of Health and Welfare (MHW), from which will emerge six new health and medical research institutes.

The main aim of the MHW reorganization is to bring together institutes with common or related roles. For example, four separate institutes involved in public policy research, covering topics such as public health and population problems, will be combined into two.

Public criticism of the failure of the reform plan to go as far as some feel necessary has further weakened the coalition government, already being torn apart by a split within the Social Democratic Party, headed by the prime minister. But administrative reform is likely to be continued by future governments, and the *tokushu hojin* may therefore still experience more attacks.

David Swinbanks

ESO chief pledges to fight Chilean telescope ruling

Garching. Riccardo Giacconi, director of the European Southern Observatory (ESO), said last week that he plans to defy a court order to stop building work on the observatory's next big project, the Very Large Telescope (VLT), sited at Mount Paranal in northern Chile, unless directly threatened by the Chilean government.

If such a threat materializes, said Giacconi, he plans to take the government to international court for breach of contract. His pledge reflects frustration that, despite two years of apparently successful negotiations with the Chilean authorities, a bitter legal dispute over the ownership of the land on which it is to be built remains unresolved (see *Nature* 368, 676; 1994).

The VLT, which will be the world's largest optical telescope when it is completed, is expected to cost around DM463 million (US\$303 million).



Giacconi: prepared to fight government

But its future continues to be threatened by the claims of a local family to the land, which was given to ESO by the government in 1988.

Two years ago the family began legal action to secure recognition of its claim. But

ESO has always maintained that, as an international organization, it has immunity from local law. It has entered a chain of appeals and counter appeals with the Chilean courts, while the government, with which ESO has an agreement dating back to 1964, has stood aside.

Last spring, ESO stopped construction work after an injunction was brought against the construction company Skanska-Belfi. Giacconi says that this was a very expensive gesture of goodwill which will not happen again. "It would take the police or the army to stop us now," he says.

A year ago, frustrated by the endless problems in Chile, ESO was considering transferring the location of the VLT to an alternative site in Namibia. But investment at Mount Paranal is now too great for this still to be an option.

But ESO does have some cause to feel optimistic. Australian scientists have asked their government to become an ESO member state in order to gain access to the VLT facility. The extra money from expanded membership would allow ESO to restore the VLT interferometry for completion in the year 2000 (it has been delayed to save money).

Alison Abbott

Indirect cost rules seek to deflect threat of 'capping'

Washington. The Clinton administration has moved to deflect an imminent congressional attack on the government's funding of indirect university research costs by publishing new rules standardizing the basis on which they are assessed.

But some university researchers fear that the new rules — published in the *Federal Register* on 6 February — lack the clarity and simplicity needed to convince Congress that the \$3 billion reimbursed to universities by the federal government each year to cover such costs is being properly spent.

Congress has long been concerned about the differences in overhead costs charged by universities, which can vary from one-third to almost two-thirds of direct research costs. Of \$12 billion spent by the federal government on university research this year, \$3 billion was supposed to cover indirect costs. A budget proposal attached to last November's Republican election manifesto, the *Contract with America*, pledged to cut this by 10 per cent.

The elite private research universities tend to charge the most, and thus fear a congressional attempt to 'cap' the indirect cost rate. Public universities often pay for their facilities out of state funds, and therefore charge lower overhead rates.

The administration's new proposals, worked out after extensive consultation with the research universities, would set benchmarks for the cost of buildings and utilities in different regions of the United States, to be used to set limits on the costs researchers could recover. They would also set tighter rules for recovering the depreciation costs of new buildings.

The rules would also — somewhat optimistically — eliminate the term 'indirect costs' from official discussion, referring instead to 'research administration' and 'research facilities' costs.

The proposals remain controversial. Sam Silverstein, chair of the department of physiology and cellular biophysics at Columbia University, New York and president of the Federation of American Societies for Experimental Biology (FASEB), the biomedical research lobby group, says that the proposals are incomplete and too complex. Even though he feels that "the administration is broadly on the right track", he predicts the proposals will prove "too little and too late" to avoid "misdirected and harmful legislative action" in Congress this year.

But they have been welcomed by university administrators as indicating the administration's readiness to oppose the capping of indirect research costs. Interested parties have two months to comment on the administration's proposals.

C. M.

White House under fire over lack of clear priorities...

Boston. Leading US industrialists last week criticized Jack Gibbons, science and technology adviser to President Bill Clinton, for failing to set sufficiently clear priorities in the science budget for the 1996 financial year, which starts on 1 October.

Several industrial representatives attending a policy forum at Massachusetts Institute of Technology (MIT) called on the science community to accept that some areas have reached 'maturity' and should be run down, allowing more promising fields to prosper.

In particular, James Vincent, chief executive of the biotechnology company Biogen, and William Brinkman, head of physical science research at AT&T, both accused scientists — as well as Gibbons' science policy apparatus — of failing to make the kind of tough choices that the private sector has had to make in recent years.

Vincent said that scientists were expending too much "heat" on asking for more money, and not shedding enough "light" on ways in which the money could best be spent. "Everyone wants to get more of the pie, and frankly I get fed up with it," he said.

He predicted that the Republican-dominated Congress would ignore pleas for more money coming simultaneously from all fields of science. Instead, scientists should concentrate on helping Congress to identify priorities for support — and for cuts.

Brinkman said that AT&T is already cutting back on physics research, even though this had provided the basis of the recent information revolution, in order to concen-

trate on pressing problems in software and systems engineering.

He claimed that scientists are "glossing over the problem when we say we'll keep everything going and don't know where the breakthroughs will come from". Brinkman added that condensed-matter physics is now a mature field. "We can decide which fields are mature and which are not. And we haven't done it."

In contrast, however, most of the 200 scientists at the forum — organized by Charles Vest, president of MIT, and held the day after Clinton's budget proposals were released — appeared to support the Clinton administration's cautious efforts to back basic research on all fronts simultaneously.

"You have to be very careful when you think you know what frontiers we ought to be working on," said Gibbons. He pointed out, for example, that when he had worked in nuclear physics, many of his colleagues dismissed optical spectrometry as a research backwater, but that it had later played a critical role in telecommunications.

Frank Rhodes, president of Cornell University and chairman of the National Science Board, which is responsible for the National Science Foundation, expressed disappointment with the \$73-billion science budget proposed by the White House for next year, saying that it failed to specify research priorities. He also questioned the effectiveness of the National Science and Technology Council, the body set up by Gibbons to establish a national science policy.

Colin Macilwain

... as Congress finds its first victims

Washington. Budget-cutters in the new US Congress found their first victims in the science and technology budget last week, as the House of Representatives Appropriations Committee recommended rescinding more than \$3 billion in federal funds that had already been granted to several federal agencies in 1995 and earlier years. The rescission was needed to pay for a supplemental package of defence spending.

Among the casualties was the Commerce Department's Advanced Technology Program (ATP), a special target of Republicans opposed to government/industry cooperation. The appropriations panel recommended cutting \$107 million, about a quarter of the ATP's 1995 budget, effectively preventing any new industrial partnerships beyond those already existing (see *Nature* 373, 374; 1995).

Claiming that "the record of defence conversion has not been a good one", the committee also slashed the Defense Depart-

ment's Technology Reinvestment Program by \$502 million, and rescinded another \$35 million from defence conversion activities.

The panel cut \$100 million from the Department of Energy (DoE) and \$150 million from the Defense Department for environmental restoration activities not directly related to cleaning up defence laboratories. DoE's clean coal technology programme also took a cut of \$200 million out of a \$2.75 billion budget.

The National Aeronautics and Space Administration loses \$400 million earmarked for construction of two new wind tunnels. The Clinton administration included no additional money for the project in its 1996 budget request, prompting the Appropriations Committee to delete it.

The rescission bill now moves to the full House and then on to the Senate, where it is expected to be adopted. Another harsher rescission package is expected from the committee next month.

Tony Reichhardt

CNRS plays down 'embryo freezing' results

Paris. Coverage in the media of claims by a French research group that freezing embryos affects their subsequent development — and that as a result the safety of artificial reproductive procedures in humans may have to be reassessed — have led the Centre National de la Recherche Scientifique (CNRS) to take the unusual step of issuing a statement playing down the significance of the research team's conclusions.

The research was published last month in the *Proceedings of the National Academy of Sciences* (92, 598–593; 1995) and was carried out by the Paris-based groups of Pierre Roubertoux at the Laboratory of Genetics, Neurogenetics and Behaviour, run jointly by the CNRS and the University of Paris V René Descartes, and Maurice Auroux of the Bicêtre Hospital.

In the experiments, embryos collected from two strains of mice at the two-cell stage were either directly reimplanted in host mothers (the control group) or first subjected to conservation by freezing for 15 days, and then reimplanted.

The researchers found that cryopreservation caused no "major anomalies". But they did find what they claimed to be significant

differences between the two groups in several aspects of morphology and behaviour, such as the shape of the jaw and performance in activity tests.

Despite the first conclusion, the authors wrote that "substantial arguments support the hypothesis that embryo freezing can have delayed consequences". They added that this possibility "could perhaps justify a more limited use of this technique in clinical practice, until clearer conclusions about its effects in human embryos can be drawn".

Their conclusions have since received wide publicity. France's daily newspaper *Le Monde*, for example, described the results in a front-page story entitled "The risk of freezing embryos", while a news article in *Science* carried a similar headline. The newspaper reports appear to have led Jean-Louis Beaumont, a physician and a member of the French parliament, to call on Edouard Balladur, the prime minister, to introduce an immediate ban on freezing human embryos.

But the CNRS has now issued a press release, written by Roubertoux, claiming that the data in the paper did not justify the publicity given to its conclusions. The release also says that the reported "statisti-

cally significant differences" are similar to those usually observed between two different lines of mice.

The results, the communiqué add, show that any morphological or behavioural differences caused by freezing are small compared with those due to genetic inheritance, or "social and environmental variations".

Roubertoux claims that this interpretation of his research results is identical to that in the *PNAS* paper. But, although the paper does discuss the limitations of the data, it does not do so in such explicit terms as the subsequent communiqué.

Indeed, while the abstract of the *PNAS* paper asserts that the results "question the neutrality of artificial reproductive technologies", Roubertoux says he now acknowledges that none of the results give cause for concern about the safety of embryo freezing techniques.

Nevertheless he still maintains that the safety of freezing techniques used in artificial reproduction technologies has been neglected as a topic of study, and that further research is needed before the possibility that freezing may affect the development of embryos can be discounted. **Declan Butler**

France keeps up pressure for maritime research policy

Paris. The new head of France's marine research agency IFREMER promised last week to continue the efforts of his predecessor, Pierre Papon, to create a European maritime research policy.

Pierre David, at present president of the Cité des Sciences et de l'Industrie in Paris, was speaking shortly after his nomination as the new president of the agency, whose full title is the Institut Français de Recherche pour l'Exploitation de la Mer.

David is a former naval architect; he acted as adviser to the conservative prime minister Jacques Chirac on transport and urban policies during the 1970s, and later to the ministers for defence and transport. He was director of industrial policy at Aérospatiale from 1981 to 1986, and later became president of Souriau & Cie, a major electronics supplier to the defence industry.

The agency he is to head was created in 1984, and has responsibility for fundamental and applied marine research, as well as for large industrial and commercial activities. Its annual budget is about FF1 billion (US\$190 million), with a staff of 1,800.

During his six-year spell as president, Papon championed the need for a European maritime policy, and in particular developed close links with the agency's British counterpart, the Natural Environment Research Council. This has led to Franco-British cooperation, for example, on the development of optical sensors and satellite oceanography.

Papon has actively promoted ideas for a European Maritime Agency, "in which research should play a full part," and David agrees that maritime policy can "no longer be conceived at the nation-state level."

The member states of the European

biotechnology and the role of the oceans in climate change are likely to be key issues in the longer term.

But the EU's efforts are fragmented compared with those of the United States and Japan. At the European Commission, for example, five different directorates are responsible for more than a dozen maritime programmes.

Coordination of marine research at the European level is coming about in two ways. Over the past decade, bilateral and multilateral agreements have multiplied.

The Franco-Italian project Arco-Bleu, for example, aims to develop satellite tracking of shipping and coastlines to monitor pollution while last year saw the launch of *L'Europe*, a joint Franco-Italian 30-metre research catamaran.

At the same time the embryo of a European marine research programme has been created with the launch in 1989 of the EU's Marine Science and Technology Programme (MAST), whose current programme has a budget of ECU228 million.

The idea of a European Maritime Agency emerged from a joint meeting in Strasbourg in 1992 of the European Science Foundation and the commission, at which the heads of all the marine research agencies of the member states came together for the first time.

D. B.

IMAGE
UNAVAILABLE
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REASONS

Nautille maintains the tradition of Jules Verne.

Union (EU) spend around ECU850 million (US\$1.05 billion) a year on marine research and technology. The research fleet includes around 40 ships — half of which are either British or French — and two research submarines, both of them French.

The economic and political stakes are high, given that marine research affects fisheries, oil exploration, shipping, transoceanic communications and the naval components of nuclear dissuasion. Moreover, according to Papon, Europe's 15,000 km of coastline means that it needs a concerted approach to problems of the environment, while marine

E. La Couppelle/IFREMER

The role of seismology

SIR — *Nature's* leading article on the Kobe earthquake¹ referred to the lack of progress of Japan's earthquake prediction programme towards its stated goal, a point I have also raised^{2,3}. Asking "Is there nothing seismology can do?", the leading article suggested increased use of seismological data to design earthquake-resistant structures. Much work is being carried out in this area; further progress is certainly desirable.

But the most important duty of seismologists is to provide the public and government with accurate and speedy information on earthquake source parameters. Because this was not done for the Kobe earthquake, there were needless casualties and property damage.

The Japan Meteorological Agency (JMA) initially announces the 'intensity' recorded at each observatory on an arbitrary and somewhat subjective scale from 1 (barely perceptible) to 7 (violent shock). Both the public and the government seem to regard intensity, rather than magnitude and hypocentre (which are announced simultaneously, but not emphasized), as the main data characterizing an earthquake.

The use of intensity, which is an archaic parameter dating back to pre-instrumental seismology, had disastrous consequences. All telephone lines in the Kobe area were immediately knocked out by the earthquake. Because intensity data are normally transmitted by ordinary telephone lines, intensity readings from the most severely damaged areas were not immediately available. As a result, the initial JMA announcement, made 18 minutes after the earthquake, showed a highest intensity of 5 (strong shaking), at Kyoto, Hikone, and Toyooka⁴. It is remarkable that the JMA did not immediately grasp the implications of the failure of observatories at Kobe and Awaji Island to issue reports. But, in any case, the announced maximum intensity of 5 caused the government and national media, which are based in Tokyo, to underestimate the size of the earthquake. Intensity reports trickled in by radio from the most severely damaged areas, but the full extent of the damage only gradually became clear to people outside the Kobe area, including the cabinet, about 4–6 hours after the main shock.

The Kobe earthquake had a magnitude of 7.2 (JMA), and the initial data-processing showed that the hypocentre was clearly shallow. All seismologists know that an earthquake of this size at a shallow depth under a densely populated metropolitan area will cause severe damage. If the implications of the Kobe earthquake's magnitude and location had been properly explained to government author-

ities, they would have known they had to begin disaster relief operations even before receiving damage reports from the field. Such a prompt response could have saved the lives of many victims trapped in collapsed buildings, and minimized the damage caused by fires.

The initial announcement should give not only the magnitude and location but also data describing the strength of the ground motion at each observatory. But, rather than intensity, a physical parameter such as the maximum acceleration should be given.

The JMA already has elaborate bureaucratic procedures for issuing an earthquake prediction, although none has ever been issued. In contrast, as shown by the present case, the procedures for promptly notifying the government and media when a damaging earthquake does occur are inadequate and must be rethought. The CUBE (Caltech-USGS Broadcast of Earthquakes) system provides a useful model⁵ for what might be done on a national scale in Japan.

Seismologists failed to serve the public properly in the Kobe earthquake. To prevent the recurrence of such a tragedy, Japan's earthquake prediction programme should be abolished, and an entirely new programme for fundamental research in seismology, rapid dissemination of accurate earthquake parameters and research and observations in strong motion seismology should be instituted.

Robert J. Geller

Department of Earth and Planetary Physics,
Faculty of Science,
Tokyo University,
Tokyo 113, Japan

1. *Nature* **373**, 269 (1995).
2. Geller, R. J. *Nature* **352**, 275–276 (1991).
3. Geller, R. J. *Proceedings of earthquake prediction research symposium* (in Japanese), Tokyo (1994).
4. *Daily Yomiuri*, 29 January 1995.
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Salam's successor

SIR — Your article about the search for a new director for the International Centre for Theoretical Physics in Trieste contained several inaccuracies (*Nature* **373**, 182; 1994).

The search party concluded its work in March 1994. Following agreement between the directors general of the International Atomic Energy Agency (IAEA) and the United Nations Educational, Scientific and Cultural Organisation (UNESCO) and consultation with the Italian government, Dr Praveen Chaudhari, a scientist of Indian origin — not Pakistani as stated in your article —

who was one of the candidates recommended by the search party, was offered the post. He subsequently declined the offer, for personal reasons. Since then, the two directors general have interviewed other scientists whose names had been suggested by the search party. No decision has yet been taken. Your article is wrong in saying that the search party has been reconvened.

The Italian law on the transfer of administrative responsibility for the centre from the IAEA to UNESCO was approved in January. Contrary to the statement in the last paragraph of your article, financing is also now secured beyond 1998.

Finally, the former director, Professor Abdus Salam, is 69 years old, not 78.

Hans Blix

(Director General)
International Atomic Energy Agency,
PO Box 100, A-1400 Vienna, Austria

□ The information about the reconvening of the search committee was based on information provided by the IAEA. Although the Italian parliament had agreed at the time of writing to provide support for ICTP beyond 1998, the decision had yet to be published in the official gazette as required before it can become law.

Editor, *Nature*

Darwinist Lysenko?

SIR — You say (*Nature* **373**, 90; 1995) that "Neither the magazine [*The Spectator*] nor Collins appear to have remembered that Soviet-style Marxism backed Lysenko against Darwin. . .".

This is not so. Lysenko claimed to be a Darwinist, which was the 'official line'. In the purging of Bukharin, Prezent, an ally of Lysenko, accused Bukharin of "erroneous and anti-Darwinian theories" and also said that "bandits" had annihilated instruction of students in Darwinism in the Leningrad State University (Z. A. Medvedev, *The Rise and Fall of T. D. Lysenko*, Columbia University Press, 1969). Dunin said: "the enemy of the people, Bukharin, fought Darwinism. . ." Lysenko wrote a polemic "Of the distorting mirror and some anti-Darwinians," grouping them with his hated Morganist-Mendelists. Lysenko said that Darwinism was part of Marxism. He also said "[Prezent] showed me that the roots of the work I am doing lie in Darwin. And I, comrades, must confess here straightforwardly in the presence of Iosif Vissarionovich [Stalin] that to my shame I have not studied Darwin properly".

Evidently Lysenko tried to justify his nonsense by calling on Darwin.

Thomas H. Jukes

Department of Integrative Biology,
University of California, Berkeley,
6701 San Pablo Avenue,
Oakland, California 94608, USA

Polite row about models in biology

A public debate last week between Stuart Kauffman and John Maynard Smith entertained a London audience but left the status of modelling in biology ill-defined.

DISAPPOINTINGLY little blood was spilled last week at the Linnean Society of London, where Professor John Maynard Smith (Sussex) and Dr Stuart Kauffman (Sante Fe Institute) had been tempted by the London Evolution Group to a public debate on Kauffman's mathematical models of evolution. In the event, they were restrained by their innate good manners, even their mutual liking for each other; Kauffman was one of Maynard Smith's first graduate students 30 years ago (and was probably as precocious then as he is still), and even claimed (physician as he is) to have cured Maynard Smith of pneumonia contracted on a visit to Chicago.

Last week's disappointment is real, because the advertised debate concerns a real issue. Kauffman's book *The Origin of Order*, in which his argument was rehearsed, caused something of a stir on its publication 18 months ago. A "mixed reception" is what the publishers (Oxford University Press) would have said (see *Nature* **365**, 704; 1993). But last week, Kauffman would have known in advance that there were some friends among the audience. One distinguished British physicist volunteered that he had found the book to contain the only comprehensible account of the mathematics of evolution.

Kauffman's argument is straightforward enough. The principle of order is the self-organizing principle, right? And living things are self-organized entities; look how much like a cell a synthetic lysosome seems! The principles of neo-Darwinian evolution are heritable variation, the differential fitness of the inheriting individuals and then natural selection. So the central problem, to Kauffman, is to understand how self-organization and natural selection can operate simultaneously.

Nobody last week disagreed about the use of the likely number of viable offspring as a measure of fitness, but Kauffman sees the representation of the problem as what the mathematicians call a complex problem. There is a "fitness space" or "landscape" with as many dimensions as there are potentially heritable variations in which, as with complex problems in physics, there will be a very large number of local maxima, corresponding to phenotypically advantageous inherited configurations of variations. So how does natural selection, working on the variations of fitness a group of organisms has inherited, manage to beat a path to the global maximum of fitness?

Kauffman last week described a simple model of this state of affairs — a peptide with four amino acids for which the possible variations are the replacement of one amino acid by another. For what it is worth, the model is derived from one introduced by Maynard Smith some time ago (*Nature* **225**, 563; 1970). To make the problem manageable, it is supposed that the only allowable amino acids in the four-peptide are alanine and glycine.

In principle, it should eventually be possible to compile a table of how all possible variations affect the effectiveness of the peptide in its biological role, and thus to map their fitness. Using arbitrarily chosen numbers, computer modelling shows the convergence of different peptides to a common end-result.

More generally, it is even now possible to demonstrate that there are indeed local maxima on the fitness landscape, and that the landscape as a whole is the more rugged as the fitness comes to depend the more extensively on variations at different sites in the four-peptide. The greater the interconnectedness, the more peaks appear in the landscape and the more difficult it is to reach local maxima, let alone the global maximum, by random walking on the landscape.

So, says Kauffman, a smooth rather than a rough fitness landscape is a prerequisite for orderly evolution. How can that be achieved? Co-evolution is one way. ("If a frog evolves a sticky tongue to catch prey more effectively, the flies it catches had better evolve sticky feet.") But evolving organisms in general must change the ruggedness of the fitness landscape their successor organisms inherit.

There remain the grand questions — the great Cambrian radiation of species, for example; was there something special about the fitness landscape of organisms then? Kauffman is a confessed devotee of the arguments (largely due to Per Bak) of the critical behaviour of self-organized systems, as in the formation of avalanches on the sides of a sandpile to which grains are added one at a time. The sandpile of self-organized living creatures must have reached a critical condition in the early Cambrian, spawning avalanches on its sides, each a new species or a genus.

Maynard Smith, an electrical engineer by background, a graduate student of J. B. S. Haldane and a modeller on his own account, is not one to deny mathematics a place in biology. Last week, he

asked that "biologists should know a limit-cycle when they see one" and know the causes of bifurcation in the evolution of chaotic systems. By way of illustration, he noted the apparent doubling of the number of segments in *Drosophila* (from seven to $7 \times 2 = 14$), suspecting that "*Drosophila* must have been reading my book".

Maynard Smith accepts the need for a relatively smooth fitness landscape, as defined, if evolution is to happen. But he could not follow Kauffman's supposition that self-organizing systems need external information to assemble, and suspected that Kauffman's view that evolving organisms influence their fitness landscape with a view to future evolution smacks of the Gaia hypothesis. The essence of his polite public dissent is simply put: the modelling of *The Origin of Order* notwithstanding, "We still do not know whether, if the environment were to stay constant, evolution would come to a stop".

Meanwhile, it is clear that Maynard Smith is not a devotee of Per Bak and his sandpiles. Among other things, he wondered what could be the correlate in the real world of the stream of sand-grains that eventually make the pile unstable. He also complained of an article in *Nature* (by this writer) "with punctuated equilibrium in the title" (*Nature* **371**, 197; 1994). That concerns numerical simulations of evolutionary models in which the fitness of co-evolving species is determined by random-number generators under particular rules. One conclusion is that it is possible to write the rules so that the initial state of the assemblage of species does not affect the outcome; another is that there can be circumstances when the system becomes unstable and several independent species emerge. Of this simulation and the sandpiles (as a model for real biology), Maynard Smith says: "I just find the whole enterprise contemptible".

What last week's audience made of the exchange is anybody's guess. Many beers later, the two protagonists did agree to try to define what their disagreement really is. After careful thought, Maynard Smith announced, "You see, Stu, I don't find it *interesting*". And that, of course, is what the argument should have been about. What is the place of modelling in biology? Can a model be heuristically valuable even when it entails only a sketchy correspondence with the real world? Maybe the Linnean Society should return to that charge.

John Maddox

Splitting molecular hydrogen

Richard Cammack

SINCE the oil crisis in the early 1970s, biological hydrogen production has been a goal for research into environmentally friendly sources of energy¹. Central to these investigations is the enzyme hydrogenase^{2,3}, and the first structure of a hydrogenase to be determined is presented by Volbeda *et al.* on page 580 of this issue⁴. It shows a hydrogen-binding site, which appears, unexpectedly, to be a binuclear cluster of nickel and iron. It also reveals the pathways by which electrons and protons may be transferred in and out of the active site.

The hydrogenase from the bacterium *Desulfovibrio gigas*, which is found in marine and freshwater sediments, is particularly interesting from the point of view of biological function, because it catalyses both hydrogen production and consumption⁵. Hydrogen is produced when the cells are growing as a result of fermentation of pyruvate, and this hydrogen may be passed on to other bacterial species such as methanogens, which use it to produce methane. Alternatively, *D. gigas* can consume hydrogen as a fuel, with the oxidant being sulphate rather than oxygen (incidentally, this reaction is significant in the corrosion of metallic iron by sulphate-reducing bacteria). As the enzyme was known to contain nickel and iron-sulphur clusters, it was termed a Ni-Fe hydrogenase³, a name that has proved to be singularly appropriate.

The catalytic site that emerges from the crystal structure of hydrogenase clearly shows two peaks of electron density. One of these is most probably nickel, and the other metal, X, has been tentatively identified from its scattering properties by Volbeda *et al.*⁴ as an iron atom. Generally, spectroscopic studies on the Ni-Fe hydrogenases have been interpreted in terms of an isolated nickel active site, but there have been suggestions that an iron atom is also present^{3,6}. In the refined structure, the nickel atom is coordinated by four cysteine ligands from the protein. The second metal atom is attached to just two bridging cysteines, with three or four other unidentified non-protein ligands. It should be noted that the form of the enzyme that was crystallized is in the 'unready' state, which requires a slow, reductive activation process, during which an internal reorganization probably takes place⁷. Therefore further study will be needed to establish the arrangement of the nickel site in the active enzyme.

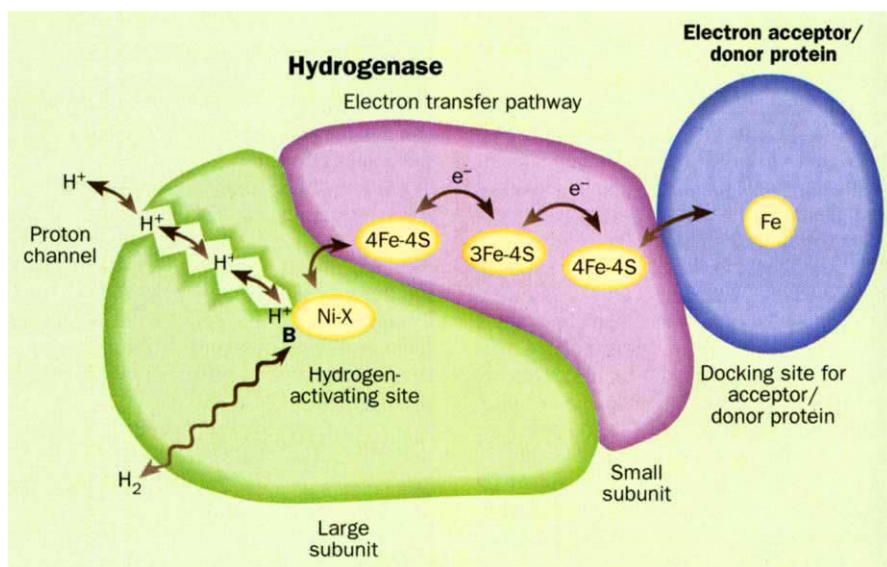
There are only four types of nickel-containing enzymes known⁸, and three of these are involved in the metabolism of gases: hydrogenase, carbon monoxide oxidoreductase, and methyl-CoM reductase

(which participates in the biosynthesis of methane); the other is the hydrolytic enzyme urease. Structurally, the different types of nickel enzyme do not seem to be related.

The insertion of nickel into the nascent protein has been investigated by studies of accessory genes for the biosynthesis of

unknown structure. It now appears that the two types of hydrogenase have in common a cluster of metal ions, at least one of which is iron. The importance of iron in the reaction of enzymes with gases is underlined by the existence of iron-containing nitrogenases without molybdenum¹¹, and the observation that in carbon monoxide oxidoreductase the carbon monoxide seems to bind to iron rather than to nickel¹².

A crown of acidic amino acids on the surface of the small subunit has been



The structural components involved in the hydrogenase mechanism. The active site is buried inside the large subunit. The electron pathway comprises three iron-sulphur clusters, arranged in a line from the nickel-containing active site to the surface of the small subunit, where there is a potential docking site for a c-type cytochrome. The proposed proton channel comprises a chain of proton-carrying amino-acid residues leading to the surface of the large subunit. Hydrogen, the third component of the reaction, being a small, diffusible molecule, probably does not require any special channel.

Ni-Fe hydrogenases. Several specific gene products are required for uptake and insertion of the nickel, after which a short peptide is cleaved from the carboxy terminus of the large subunit⁹. The structure shows that after this cleavage, the remaining C-terminal part of the sequence, containing two of the ligands to nickel, tucks snugly inside the protein, effectively locking the nickel into place.

The mechanism of the reaction of hydrogenase with hydrogen has been shown by isotope exchange to involve heterolytic cleavage to a proton, H⁺, and a hydride, H⁻. There are a number of groups that could act as the base (B in the figure) to accept the proton, including an arginine, a histidine, the thiolate ligands, and possibly hydroxide ions. The hydride could bind to a metal atom, or as a bridging ligand, as Volbeda *et al.* point out⁴. Mechanistically, an iron-nickel binuclear cluster is an attractive model for the active centre. There is another class of hydrogenases that do not contain nickel (the Fe hydrogenases)¹⁰, in which the active site is a cluster of iron atoms, of

identified as a potential docking site for a protein such as cytochrome c₃, which acts as the source or sink for electrons. Between this site and the catalytic centre, three iron-sulphur clusters in hydrogenases are neatly arranged as 'stepping stones' for electrons. It is known that electron transfer can occur easily between

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iron-sulphur clusters, over distances of 1.0–1.5 nm. However, the middle iron-sulphur cluster in the chain is the [3Fe–4S] cluster, which has a considerably more positive midpoint potential (–35 mV) than the two [4Fe–4S] clusters (–290 and –340 mV respectively)¹³. This means that it is reduced for most of the time during the enzyme reaction, which should limit the rate at which it can accept electrons. Such an arrangement of clusters might serve as a means of regulating either the

rate or the direction of electron flow.

Hydrogenases are able to react smoothly and rapidly with hydrogen gas without the help of expensive metals such as platinum: the way in which they manage to do this is of great interest to chemists designing catalysts for the same reaction, for example in making fuel cells. □

Richard Cammack is at the Centre for the Study of Metals in Biology and Medicine, King's College, London W8 7AH, UK.

BATTERY TECHNOLOGY

Challenge of portable power

Bruno Scrosati

On page 598 of this issue a group at the Tokyo University of Agriculture report on a family of composite organic electrodes for possible use in the development of high-energy-density batteries¹. Very many papers dealing with new battery materials are now appearing in the technical literature. Given the great variety of batteries on the market, why do we need new ones?

People who buy battery-powered devices are often disturbed by three facts. The battery is generally not included; the battery pack is heavy and occupies a large fraction of the device's volume; and the battery does not last long enough. In consumer electronics, one faces a technological paradox: whereas electronic circuitry has progressed dramatically in the last few decades, the batteries that power the devices are based on concepts dating from a century ago. Changes in construction and packaging aside, the most common battery for portable electronics, the nickel-cadmium type, operates on an electrochemical reaction proposed by W. Jungner in 1899. Primary alkaline batteries are direct descendants of the 1866 Leclanché zinc-carbon cell².

A similar situation applies to lead-acid car batteries, whose chemical process was announced in 1859 by Gaston Planté. Now the chief power source used for ignition in internal combustion engines and for propulsion in electric vehicles, these batteries may be adequate for the former application, but they store too little energy to be truly practical for the latter. Standard lead-acid-powered electric vehicles achieve an average driving range of only 50–60 miles at a cruising speed of 50 miles per hour. So, despite the fact that zero-emission cars would be welcome in most urban areas, their development has been limited to a few demonstration prototypes.

Simply put, conventional batteries do not meet the power requirements for either consumer electronics or electric vehicles. Their principal problem is that

their electrode combinations have limited specific capacity (ampere hours per gram of reactants), which is reflected in their relatively low energy densities (watt hours stored per weight or per volume of the battery). As shown in the figure, which illustrates the relationship between gravimetric and volumetric energy density, batteries such as lead-acid and nickel-cadmium are capable of only 30–70 W h kg^{–1} and 50–150 W h l^{–1}. Improved design and electrode configuration may raise the energy content a little. But a major increase can only be achieved through the development of new, advanced batteries that can at least double the energy values of the conventional systems.

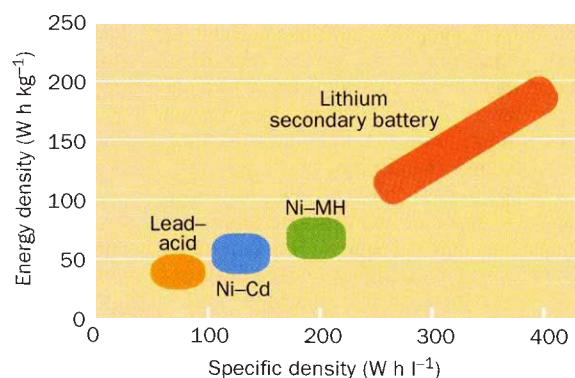
This goal requires electrode materials with higher specific capacities than nickel or lead. Research has focused on the alkali metals, particularly lithium, as it is the easiest to handle and, most significantly, is the lightest and most electronegative of the family.

In the most general scheme, a rechargeable lithium battery uses lithium metal as the negative electrode, a nonaqueous electrolyte (liquid or polymeric, see below) through which Li⁺ ions are conducted, and a Li-intercalation positive electrode. The positive electrode is structurally the most elegant and generally has an open structure capable of accepting and releasing lithium ions to form a composition of the type Li_xA_zB_y. Good examples are the transition metal dichalcogenides (such as TiS₂) and oxides (such as V₆O₁₃), which have channels through which the guest lithium ions can move rapidly^{2,3}. Common examples of liquid electrolytes are solutions of lithium salts in organic solvents. Alternatively, polymeric membranes may be formed by the solvation of lithium salts in high-molecular-

weight polymers or by the trapping of liquid solutions in a polymer matrix, allowing the construction of batteries in appealing thin-layer configurations⁴.

In principle, lithium batteries are capable of providing the high energy densities required by modern devices (see figure). This is where the new work by Oyama *et al.*¹ fits in. The authors demonstrate the performance of a composite organic cathode based on dimercaptan and polyaniline. In combination with a lithium metal anode, this leads to high values of gravimetric energy density. The cells can be recharged (Oyama *et al.* put theirs through over 100 cycles, although with some loss of capacity after about 30 cycles). The secret seems to lie in the intimate mixing of the two cathode components at the molecular level by depositing them together from solution; cathodes of the same composition manufactured from pressed powders performed less well.

However, high energy content, although important, does not in itself make a battery suitable for practical ap-



Gravimetric versus volumetric energy density for various battery types. Derived from ref. 10.

plications. Other essential characteristics are long cycle life, reliability and safety. Unfortunately, the cycle life of lithium metal anode batteries is generally limited by corrosion reactions occurring at the lithium/electrolyte interface. These reactions may ultimately lead to total cell failure and also pose a serious safety hazard, fire for instance.

Much effort has been devoted to developing batteries that would combine the energy content of lithium anode batteries with the cycle life and reliability of nickel-metal hydride cells. Currently, the best approach appears to be to replace the lithium metal anode by a second insertion compound, such as Li_nC_m, that is capable of accepting and exchanging large quantities of lithium ions. Rather than plating and stripping lithium metal, the electrochemical process at the negative electrode is the uptake of lithium ions during charging and their release during discharge. The total electrochemical process involves the cyclic transfer of *x* equivalents of lithium ions between the two

insertion electrodes. Such unconventional electrochemical cells are effectively concentration cells in which lithium ions 'rock' from one electrode side to the other; accordingly, they have been termed rocking-chair batteries.

The concept of lithium rocking-chair cells is not new; it was proposed in the late 1970s^{5,6} and practically demonstrated in the early 1980s^{7,8}. However, it has gained renewed attention following the success of Japanese industries in exploiting the idea for large-scale production. The Japanese versions use carbon or graphite (Li_xC_6) as the negative 'lithium sink' electrode and LiCoO_2 as the 'lithium source'. The preferred electrolyte is a solution of lithium hexafluorophosphate in a mixture of ethylene carbonate and dimethylcarbonate as solvent. This work has triggered interest throughout the world, and many companies are producing carbon-based lithium rocking-chair batteries (also known as lithium-ion or swing batteries). Full-scale commercial production of lithium rocking-chair batteries with energy densities exceeding 100 Wh kg^{-1} and 250 Wh l^{-1} , cycle life of about 1,200 deep cycles, and excellent safety records is under way. Progressively, lithium rocking-chair batteries are starting to replace conventional batteries in cellular phones, video recorders and laptop computers⁹.

The present achievements, however, are far from being the ultimate answer to the technological request. Rocking-chair batteries still need higher energy densities and lower cost of manufacture, and it would be better to replace the liquid electrolyte with an ionically conductive membrane in order to realize flexible plastic batteries.

The success of the rocking-chair idea does not necessarily mean that lithium metal batteries are out of the picture. On the contrary, careful attention should be paid to improving the lithium interface stability, enhancing the battery cycle life and — as Oyama *et al.* demonstrate here — to characterizing novel positive electrode materials. □

Bruno Scrosati is in the Department of Chemistry, University 'La Sapienza', Piazzale A. Moro 5, 00185 Rome, Italy.

The molecular explosion

Henry Gee

BUSHY multifurcations are the bane of every systematist looking for neat, dichotomously branching phylogenies. But Philippe *et al.* have turned this problem into an ally¹. In molecular phylogeny, multifurcation reflects an inability to resolve close cladogenetic events (that is, divergence of evolutionary lineages), especially if they are remote in geological time. But by estimating the inherent resolving power of 18S ribosomal RNA sequences, and calibrating a molecular clock against the fossil record, Philippe *et al.* provide molecular corroboration for the Cambrian 'explosion', the classic multifurcation event when virtually all metazoan phyla burst into being between 540 and 520 million years ago.

The method is ingenious. Philippe *et al.* start with a phylogenetic tree of the metazoa as deduced from 69 complete 18S rRNA sequences. Fast-evolving sequences are then weeded out, as these can distort phylogenies in which the interest lies in extremely ancient branching events. Fast-evolving sequences tend to stick together by virtue of speed rather than phylogeny, explaining why *Drosophila* and *Aedes* find themselves in the fast lane, next to nematodes, rather than with other insects.

Fast-evolving sequences may also conspire to lower what is termed the 'bootstrap proportion' (BP). This is the number of times a given node in the tree is supported over the total number of nucleotide resamplings carried out (in this case 1,000), expressed as a percentage. Low BPs mean low support for individual branches, which gives plenty of scope for the introduction of a new taxon to change the entire topology of the tree.

Philippe *et al.* advocate ruling out BPs of less than 95%. Weeding, unfortunately, bars many interesting taxa from consideration (including, not surprisingly, *Drosophila* and nematodes), but it still leaves 55 sequences distributed across most of the major phyla.

The new tree provides unequivocal support, in the form of a BP of 100%, for a deep split between diploblasts (animals such as jellyfish which have two layers of cells) and triploblasts (which additionally have a mesoderm). Animals of possible diploblastic grade are believed by many — but not all — palaeontologists to characterize the pre-Cambrian Ediacara fauna, which, at 570–555 million years old, significantly antedates the Cambrian explosion itself. The tree is equally robust in its support for a deep split between acoelomate flatworms and coelomates (animals with mesoderm-derived cavities), which is more contentious.

But nodes reflecting events within the Cambrian explosion itself are, as expected, generally less well-supported, and this support declines with increasing phyletic inclusiveness: the monophyly of protostomes, one of the two main divisions of coelomates, is supported in just three-quarters of all resamplings (a BP of 75%), but the monophyly of arthropods commands a solid 98% and vertebrates an unimpeachable 100%. But the monophyly of deuterostomes, the other major coelomate division, musters a debatable 69% and that of coelomates as a whole only 49%, less than half the resamplings.

But do these low BPs really reflect remote, closely spaced cladogenetic events, or are they simply an artefact of insufficient data? Philippe and colleagues attack this important issue with the application of a new method developed by their group². Expressed simply, this measures the effect on BP of increasing the number of nucleotides used in the analysis. So if the number of available, variable nucleotide sites produces a low BP for the node of interest, how many additional nucleotides would you need to force the BP above 95%; that is, how many are necessary to bolster the node with adequate statistical support?

Bear in mind a few figures in view of what comes later: the present analysis is based on an alignment of 1,474 nucleotide sites of which 708 are variable and 486 phylogenetically informative. Vertebrate and arthropod monophyly (BPs of 100% and 98%) are already well supported by this data set, the lower value of arthropod monophyly perhaps reflecting the greater relative age of the constituent groups.

But protostome, deuterostome and coelomate monophyly are different. Calculations show that the current BP values (respectively 75%, 69% and 49%) are about the best that can be managed with the present data set. Getting the BPs up to 95% would require more sequence information — a total of 1,300 variable nucleotides for protostomes, 2,100 for deuterostomes and 2,600 for coelomates, far in excess of what is available (or even possible, given that the analysis already uses complete 18S rRNA sequences). This suggests that no matter how much data one throws at the problem, there are definite limits on the ability of information derived from 18S rRNA to resolve closely spaced cladogenetic events. But how close?

This is where Philippe *et al.* bring in one or two justifiable assumptions about the molecular clock, and a few well-supported palaeontological markers, to work out the number of nucleotides of a given molecule

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required to discriminate between two cladogenetic events occurring in any given time interval.

The result is a plot of number of the variable sites against time. The plot is a hyperbola — well-spaced cladogenetic events are easily resolved with relatively little data, but the number of variable sites required rises steeply as intervals shorten. With 200 variable sites of 18S rRNA, resolution is no better than 140 million years. With 708 variable sites from complete 18S rRNA sequences, as in this study, the limit of the resolution of 18S rRNA sequence data is 40 million years. Adding the complete sequence of 28S rRNA — to give a total of around 1,500 nucleotides — would permit a resolution of 19 million years. To get down to one million years (very nearly the scale of current palaeontological and stratigraphic

sampling) one would require 28,000 variable nucleotides, beyond the limits of present practicality.

The 40-million-year limit on the resolving power of 18S rRNA means that adjacent nodes supported by BPs of less than 95% probably reflect cladogenetic events that occurred within this period. Translated into real animals, this suggests that deuterostomes and protostomes diverged from a primitive triploblastic coelomate ancestor, and underwent considerable subsequent divergence, within a period of 40 million years or less. In other words, the Cambrian explosion. □

Henry Gee is an assistant editor at Nature.

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MAGMATISM

Underplating over hotspots

W. Steven Holbrook

MOST of the magmatism that creates the Earth's crust occurs in well-defined tectonic zones: mid-ocean ridges, continental and island arcs, and continental rift zones. In contrast, hotspots — sites where plumes of hot material rise from deep within the Earth's mantle — can occur nearly anywhere, affecting both continental crust (as beneath Yellowstone) and oceanic crust (as beneath Hawaii). But it is difficult to quantify exactly how much magma hotspots deliver, and what that magma is composed of, because of the likelihood that a substantial portion of the material is not erupted but intruded as a deep-crustal 'underplate', where its presence can be inferred only by indirect geophysical methods. On page 600 of this issue¹, Caress *et al.* settle a controversy over the occurrence of crustal underplating, and in so doing raise several fundamental questions about the magmatic processes that link the Earth's crust and mantle. Their paper describes seismic refraction experiments carried out in the Marquesas Islands in the Pacific.

The controversy settled by Caress and his co-authors is whether underplating ever occurs above oceanic hotspots. The only previous modern seismic study in such an environment, conducted more than a decade ago across the Hawaiian island of Oahu, yielded ambiguous results. Although ten Brink and his co-workers interpreted the data as evidence of an underplated layer similar to that beneath the Marquesas^{2,3}, an alternative view of the same data concluded that no such layer was required⁴. The results of Caress *et al.* confirm the existence of crustal underplating above oceanic hot-

spots. Moreover, the rapid increase in seismic velocity with depth requires that the composition of the crust changes vertically. Thus neither the volume nor the composition of hotspot magmatism could have been accurately predicted from the surface lavas alone.

Because underplating is defined as magmatic material added to the base of the Earth's crust, one of the main difficulties in quantifying it — indeed in identifying it — has been to distinguish underplating from the pre-existing crust. This problem is particularly acute beneath continents, the crust of which is thick (25–45 km) and laterally heterogeneous. The Marquesas Islands, because they are built on thinner (6–8 km), simpler oceanic crust, offer a natural laboratory in which the structure of the pre-existing crust, and therefore of the underplate, can be confidently determined. Moreover, because the Marquesas hotspot lies within a large tectonic plate, magmatism there is not increased by a mid-ocean-ridge spreading centre, as it is at Iceland for instance.

The surprisingly large volume of magmatism beneath the Marquesas Islands — 950,000 km³, nearly two-thirds of which resides in the underplated zone — implies that the Marquesas hotspot is about as strong as the Hawaiian hotspot. The thickness of magmatic material emplaced above an intraplate hotspot depends on the strength (flux) of the plume and the speed at which the overlying plate moves past. Because the Hawaiian and Marquesas Islands both lie on the Pacific Plate, the equivalence of magmatic thickness beneath them (about 17 km) implies that the plume fluxes are roughly equal.

Previous measures of plume flux, however, based on estimating the amount of buoyant plume material needed to uphold the seafloor swell around volcanic chains, had designated the Hawaiian hotspot as the strongest on Earth, with a flux 1.3–2.5 times that of the Marquesas^{5,6}.

One way around this conclusion is if the high-velocity material contains a substantial proportion of pre-existing mantle. Velocities of 7.9 km s⁻¹ correspond to ultramafic compositions⁷ and are usually interpreted as upper mantle⁸. Caress *et al.* assign this zone to the crust because of the strong reflector at its base, which they interpret as the crust–mantle boundary (Moho). If, however, the high-velocity material largely consists of pre-existing mantle material, then the 17-km crustal thickness is an overestimate, and the estimate of Marquesas plume flux decreases accordingly. Such an interpretation, though, provides no ready explanation for the strong Moho reflector.

If the interpretation of Caress and colleagues is correct, it raises the question of why the underplate has such high velocities and, hence, a different composition from the upper crust. Are the upper crust and underplate derived from primary magmas of different compositions, which rise to different levels of neutral buoyancy? Or are the upper and lower crust formed by crustal fractionation of a single primary magma? Other unanswered questions brought to light by the Marquesas study are whether underplating occurs contemporaneously with the extrusive volcanism¹, or during a later episode related to flexural stresses induced by the overlying volcanic load². How is the underplate emplaced — is it as vertical dykes or horizontal sills? And how is the delivery and emplacement of melt affected by a nearby spreading centre?

These questions will surely be the subject of future geological and geophysical investigations. Meanwhile, the seismic results from the Marquesas islands have confirmed the intuition of many geoscientists that the lava erupted from a hotspot volcano is only the tip of the iceberg. □

W. Steven Holbrook is in the Department of Geology and Geophysics, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543, USA.

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Tense spindles can relax

Andrew W. Murray

LIKE fine wines, clever experimental approaches often improve with age. For more than 25 years, Bruce Nicklas and his colleagues have been exposing the rules that ensure accurate chromosome segregation by micromanipulating chromosomes in insect spermatocytes undergoing the first meiotic division. The culmination of their odyssey is a report on page 630 of this issue¹, which shows that cells monitor mechanical forces on the chromosomes to determine whether the spindle has been correctly assembled.

Cells care about accurate chromosome segregation because the wages of error are cell death, birth defects and cancer. Many cells with missing or extra chromosomes die, but if they survive they can damage the organism that harbours them. If meiosis produces an egg with an extra copy of chromosome 21, the child that develops from the egg will suffer from Down's syndrome, whereas mitotic errors that produce cells with missing or extra chromosomes play an important role in the genesis and progression of cancer. The key to chromosome segregation is the interaction of the chromosomes with the microtubules that radiate out from the poles of the mitotic and meiotic spindles. This interaction is mediated by the kine-

tochores, specialized protein complexes assembled on the centromeric DNA. Correct segregation requires that kinetochores destined to segregate from each other must attach to opposite poles of the spindle, and anaphase, the act of chromosome segregation, must not begin until all the chromosome pairs have achieved this bipolar orientation.

In 1969 Nicklas and Koch used grasshopper spermatocytes to show that tension at the kinetochore guided chromosome pairs towards bipolar orientation². They used a fine glass needle to detach one kinetochore from the microtubules of the spindle and then guide the kinetochore into the other half of the spindle (*a* in the figure). When this kinetochore reattached to microtubules, it produced a chromosome pair that had both kinetochores attached to microtubules originating from one spindle pole. Unlike bipolar orientation, this monopolar attachment is unstable with one of the two kinetochores rapidly losing its attachment to the spindle pole. If this kinetochore reattaches to microtubules originating from the pole it was just connected to, monopolar attachment is restored and is once more unstable. But if the free kinetochore attaches to microtubules from the

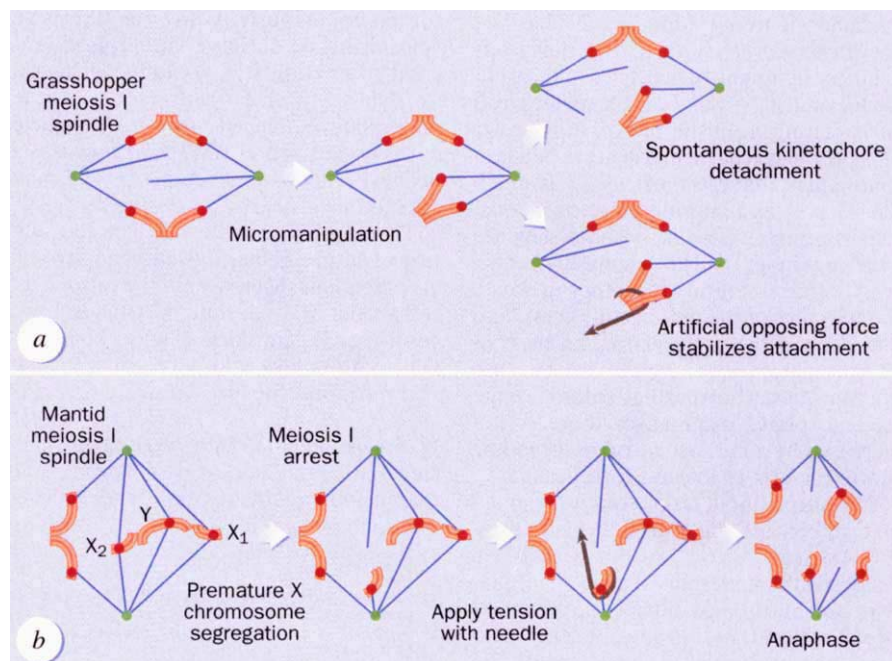
opposite pole, bipolar orientation is restored and is stable.

The important difference between the two orientations is the tension at the kinetochores. In bipolar attachment the linkage between the two members of the chromosome pair links the kinetochores and allows tension to develop when they pull against each other, whereas the kinetochores of a mono-oriented chromosome pair both pull in the same direction. To demonstrate the role of tension, Nicklas and Koch used the manipulation needle to pull against the two kinetochores of a mono-oriented chromosome pair. As long as tension was maintained, the mono-oriented chromosomes were stable.

The difference in stability between monopolar and bipolar attachment ensures that all the chromosome pairs will eventually align themselves correctly on the spindle. In many cells a checkpoint holds the cells in mitosis until all the chromosomes are properly aligned. The existence of this spindle-assembly checkpoint was first suggested by the mitotic arrest induced by microtubule depolymerizing drugs³, and was confirmed by the isolation of budding-yeast mutants that allow cells with defective spindles to enter anaphase^{4,5}. The timing of mitosis in tissue culture cells strongly suggests that the checkpoint monitors the attachment of kinetochores to microtubules; although the total duration of mitosis is highly variable, the interval between the attachment of the last kinetochore to microtubules and the onset of anaphase is constant⁶.

The latest micromanipulation experiments¹ now suggest that cells monitor kinetochore attachment by detecting tension at the kinetochore. The spermatocyte donors for this work were praying mantids, which have an unusual arrangement of sex chromosomes. During mantid evolution the single X chromosome was broken into two smaller chromosomes X_1 and X_2 , which pair with and then segregate away from the single Y chromosome at meiosis (*b* in the figure). Thus instead of being a chromosome pair, the X and Y chromosomes form a chromosome triplet that aligns on the spindle with the two X kinetochores attached to one pole and the Y kinetochore attached to the other. In about 10 per cent of spermatocytes one of the X chromosomes separates prematurely from the rest of the triplet and takes up a position close to the pole it is attached to⁷. These abnormal cells remain in meiosis I, do not enter anaphase and eventually degenerate. Li and Nicklas¹ show that the unattached X chromosome activates the spindle assembly checkpoint because it is not under tension: pulling on the unattached chromosome with a needle, and applying tension to the kinetochore, allows the cell to enter anaphase.

Kinetochore tension is the regulator *du*



Tension controls two aspects of mitosis. *a*, Micromanipulation of grasshopper spermatocytes creates a chromosome pair with both kinetochores attached to one pole. This attachment is unstable because the kinetochore microtubules detach from the spindle pole and then depolymerize¹⁴. Monopolar attachment is stabilized by applying force on the chromosomes using the micromanipulation needle. *b*, Kinetochore tension regulates the onset of anaphase in praying mantid spermatocytes. Premature separation of one of the X chromosomes causes arrest in meiosis I, but this arrest is overcome by applying tension to the errant chromosome. Kinetochores are indicated in dark red, spindle poles in green and microtubules in blue.

jour for many aspects of spindle behaviour. As well as the micromanipulation experiments that reveal its involvement in controlling chromosome attachment and the spindle-assembly checkpoint, high-resolution microscopy of mitotic chromosome movements indicates that tension may also regulate the direction of kinetochore movement along microtubules⁸. What are the molecular targets of this physical force, and how do they control an enormous and highly dynamic structure? Two advances suggest that, finally, these are tractable problems. The first advance stems from genetic and functional analysis of chromosome segregation in yeast, which has identified kinetochore proteins and the DNA sequ-

ences they bind to⁹⁻¹². The second comes from observations made with a monoclonal antibody that recognizes a labile protein phosphorylation¹³. In mitotic tissue culture cells the antibody recognizes only the kinetochores that have not attached to the spindle, or have recently attached (and are probably not yet under tension). Identifying this phosphorylated protein should help determine how tension regulates so many aspects of spindle behaviour, and usher in an exciting new era for students of mitosis. □

Andrew W. Murray is in the Departments of Physiology and Biochemistry and Biophysics, University of California at San Francisco, San Francisco, California 94143, USA.

task requiring rhyme judgements on pairs of visually presented nonsense words activated cortex in the inferior gyrus of the frontal lobe. This activation was unilateral in males but was bilateral in their female subjects. Activation of the inferior frontal gyrus was not found in two other tasks: matching consonant strings for letter case, and judging whether words belonged to the same semantic category. As neither of these tasks requires the generation of phonological representations, Shaywitz *et al.* conclude that the frontal activation in the rhyme-matching task reflects a cognitive operation necessary for phonological processing, and that this operation is more strongly lateralized to the left hemisphere in males.

Of the issues raised by these findings, perhaps the most important concerns the functional role of the right-sided activation that distinguishes the two sexes. Activation of the left inferior frontal gyrus in language tasks has been reported in studies that have used a different neuroimaging technique — positron emission tomography — to identify task-dependent changes in cerebral blood flow, and has been assumed to reflect the coding of information in articulatory form^{9,10}. In addition to their role in speech production, articulatory representations are thought to be important in tasks that require the explicit comparison of phonological features^{9,10}, of which the rhyme-matching task of Shaywitz *et al.* is an example.

Do these findings mean that the left and right inferior frontal gyri of females are equally involved in articulatory coding or, indeed, in any other language function? This conclusion would be premature for at least two reasons. First, and most obviously, the results need to be shown to generalize to other tasks that make similar demands on phonological and articulatory processing. Second, functional neuroimaging data cannot distinguish between brain activity that is necessary for a task to be performed and activity which, although correlated with the task, has no causal role in its performance. To make this distinction, results from neuroimaging must be combined with those obtained by other methods, notably investigation of the effects of lesions. Although lesion studies present interpretational difficulties of

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NEUROBIOLOGY

La différence vive

Michael Rugg

THE idea that language is represented more asymmetrically in the brains of men than of women gains fresh support from a functional magnetic resonance imaging study described on page 607 of this issue¹ by Shaywitz *et al.* It appears that activation of the inferior frontal cortex during a phonological matching task is lateralized to the left hemisphere in males, but occurs bilaterally in females.

These findings add to a literature on the cerebral basis of language which extends back into the mid-nineteenth century. Since that time the findings of numerous studies, using a wide variety of methods, have converged to suggest that in most individuals verbal functions depend heavily on specialized cortical regions of the left hemisphere. In contrast, many non-verbal functions, notably those associated with spatial skills, tend to be lateralized to the right hemisphere. It remains unclear, however, to what extent this pattern varies systematically between different groups.

The proposal that the two sexes differ significantly in their patterns of functional lateralization, with language lateralization being more complete in men than in women, is a source of particular controversy. The most direct evidence for this proposal comes from reports that males have a higher incidence of aphasia after a lesion to the left hemisphere than do females, and show larger discrepancies

between measures of verbal and non-verbal intelligence after a lesion to either hemisphere². But other investigators have either failed to find a relationship between sex, laterality of lesion and aphasia³, or have accounted for the relationship in terms of sex differences in language organization within the left hemisphere⁴. And if it is assumed that women are more likely than men to use verbal strategies to solve ostensibly non-verbal problems, the sex difference in patterns of intellectual impairment after unilateral brain damage can also be explained without supposing that females have weaker functional lateralization than men⁵.

Against this background, the results of Shaywitz *et al.*¹ represent a notable advance because they provide direct evidence for the differential engagement of the right hemisphere in a language task in normal male and female subjects. The study exploits recent advances in functional neuroimaging, which permit small changes in cerebral blood flow to be localized with magnetic resonance imaging^{6,7}. As a local increase in cerebral blood flow signifies increased neural activity at the same locus⁸, this technique allows brain regions that change their activity as a function of experimental condition to be identified. Compared to a control task, in which subjects judged whether two patterns shared the same visual features, Shaywitz *et al.* found that a

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their own, they remain the most direct way to test a hypothesis about the function of a region of the human brain. Therefore, it is noteworthy that the incidence of aphasia following damage to the right hemisphere appears to be no higher in women than in men^{3,4}, contrary to what would be expected if women were more dependent on the right hemisphere for language.

Nonetheless, the findings of Shaywitz *et al.* strongly suggest that future studies on

the effects of right hemisphere damage may reveal more subtle sex differences in language function than those that can be detected by clinical tests for aphasia. They also indicate that a good starting point would be patients with lesions of the inferior frontal gyrus. □

Michael Rugg is in the Wellcome Brain Research Group, School of Psychology, University of St Andrews, Fife, Scotland KY16 9JU, UK.

ARCHAEOLOGY

Flight into pre-history

Paul G. Bahn

EIGHT years ago, *Nature* carried a paper¹ and associated News and Views article² describing the discovery of an Upper Palaeolithic 'boomerang', made of mammoth tusk, from Poland. But the object was far too precious to be used for tests to see how well and how far it flew, so such tests have had to await the construction of realistic replicas. The ballistics experiments have now been carried out with plastic copies³. They show that this was a formidable weapon although, as was thought from its shape which is similar to that of some wooden specimens from Queensland, Australia, it was not a returning boomerang but a straight-flying throwing implement for use in hunting.

The boomerang, the only ivory specimen known from prehistory, is made from the end of a young mammoth's tusk; its span is 71 cm, and greatest width and thickness 6 cm and 1.5 cm respectively. It was originally thought to be about 23,000 years old, but accelerator radiocarbon dating has now assigned it to around 20,300. An exact replica was made in plexiglass, from which a copy was taken in plastic of the same specific gravity as mammoth ivory (1.8 to 1.9). The facsimile weighed about 800 g.

The first tester, an expert in the field of

throwing implements, had difficulty in throwing the boomerang. Not only is 800 g quite heavy for objects of this type, but the span and outline are unusual. The two ends are of different widths — the wider arm is clearly the gripping end, for the thinner arm, because of its greater curvature and less favourable position in the hand, could injure the thrower as its point slides through the hand. The broader point also has transverse engraved lines on its underside which afford a better grip.

The boomerang can be thrown with either the right or left hand without any loss of flight qualities: in a right-handed throw, there is a typical dive and rightward turn at the end of the flight path, caused by a strong decrease in rotation speed but not in forward propulsion. The tester threw the facsimile at an angle of 80–90°, almost horizontally; it slowly reacted to minor turbulence and brief shifts in wind speed, but its flight path proved extremely stable (whereas that of a wooden specimen, weighing only 200 g and with a span of 50 cm, was clearly influenced by wind conditions). Throwing the object horizontally prevents excessive lift. This may well have been intentional on the part of the carver, because a hunting weapon with excessive lift hinders

aiming, and game can spot it too soon.

With a following wind, the average flight path was 25–30 m; with the wind to the left or right, the average was 30–35 m; but against the wind the average path was 35–40 m. In other words, wind propulsion from behind had less effect on the distance thrown than the aerodynamic lift from a facing wind. The same results were obtained in summer and winter and in a variety of weather conditions.

Further tests were carried out by two sport-boomerang enthusiasts. In a wind strength of 2 on the Beaufort scale (a light breeze), various attempts confirmed that only a horizontal throw was practicable and sensible. The best throw achieved was 66 m, with a gentle facing wind (3 on the Beaufort scale).

For comparison, they also threw a bent wooden cudgel with a round cross-section, loaded with lead to weigh 800 g. It was easier to hold than the boomerang, but its blunt cross-section acted as a brake — it travelled only 27 m, at 18.5 m s⁻¹, whereas the boomerang went up to 66 m at 21.4 m s⁻¹. Comparisons from modern athletics are the world records for men's javelin (800 g) and discus (2 kg), which are around 95 m and 75 m respectively. For obvious reasons the testers could not try their skills on live targets (which, originally, were probably small game).

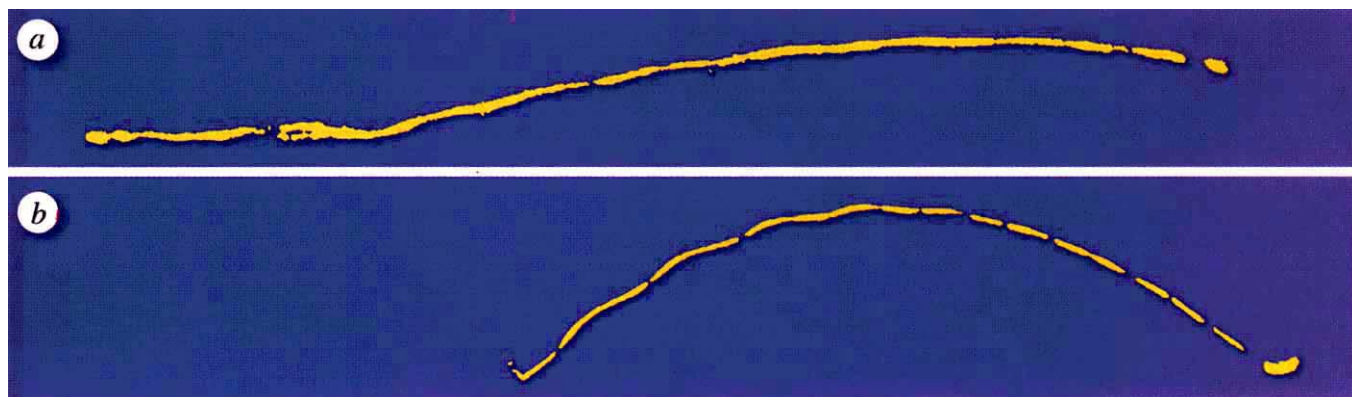
Subtle details in carving of the ivory, all apparently designed to improve the boomerang's stability and ballistic qualities, indicate that its maker knew precisely what to do. Its ingenious form points to a long tradition of making weapons of this kind; the carver was clearly drawing on long-accumulated knowledge in the working of this difficult material. □

Paul G. Bahn, a freelance writer on archaeology, is at 428 Anlaby Road, Hull HU3 6QP, UK.

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The flight path, running from right to left, of the replica 'boomerang', coated with luminous material and thrown at night (a) against that of

the similarly prepared wooden cudgel (b). The top trace is clearly the flatter and longer. (From Fig. 7 of ref. 3.)

Vibrations in the memory

Rodney J. Douglas and Kevan A. C. Martin

OF the many secrets that each of us carries in our brains, the one that we would all dearly love to know is how we remember things. How is it that *Music, when soft voices die, Vibrates in the memory*? How do we instantly pick out the individual face of a friend in a crowd, identify the aroma of freshly ground coffee, and carry out a conversation in a noisy café, all the while maintaining a coherent and stable view of the world? This complex process of segmentation and recognition may involve a temporal code based on the oscillatory activity of neural networks in the cerebral cortex. Whittington *et al.*, in a combined theoretical and experimental study of iso-

lated networks described on page 612 of this issue¹, have been able to identify a network of inhibitory neurons as the likely source of these cortical oscillations.

Investigations of memory have concentrated on one part of the temporal lobe of the cerebral cortex known as the hippocampus. The link between hippocampus and memory was established through neurological patients who, through the misfortune of disease, stroke, surgery or accident, had impaired temporal lobe function. These patients appeared normal, except that they had no memory of events that had occurred a few minutes previously. Their tragic condition — tem-

poral lobe amnesia — led to wide-ranging studies of the role of the hippocampus and related cortical structures in memory.

A central problem in memory is how information is represented by neurons². One way in which memory could work is that neurons in the cerebral cortex could learn to 'recognize' the various things that make up our world. So individual neurons might learn to recognize the smell of coffee, the texture of a cup or the taste of cream, for example. But the number of such high-level 'feature detector' neurons scales unfavourably with the number of things to be encoded, and so most theories use combinations of neurons, each of which represents a simple feature, to code for different things. Each neuron then participates in the representations of many different things.

Population-based coding schemes dra-

VISUAL NEUROSCIENCE

Reflections on transparent motion

THIS striking image exemplifies the phenomenon of transparent motion, which has long been a puzzle. Objects in the visual world are constantly passing in front of one another, yet the brain has no difficulty in distinguishing them, in this case to perceive floating leaves drifting in one direction as the tiger moves in another. Extracting such information from the jumble that reaches the eyes is not trivial. But insights into how the task is accomplished come from a paper on page 609 of this issue, in which Bradley *et al.* describe the firing properties of neurons in a part of the monkey visual system known as the middle temporal (MT) cortical area.

MT is one of the 'higher' visual areas, in that the input it receives has already been sequentially processed by 'lower' brain regions, and in particular it seems to be concerned with the detection of motion. Neurons within MT are sensitive to the speed and direction of moving objects, and each neuron has a preferred direction of motion that stimulates its maximal response. Many are inhibited by motion in the opposite direction, and this inhibition is believed to help eliminate noise and ensure an accurate representation of the moving stimulus. But many also respond to stereoscopic disparity, which corresponds to the visual plane in which the stimulus occurs. The significance of this has been a mystery, because at first glance depth and movement appear to be unconnected. Yet it also seemed unlikely that a single brain area would be

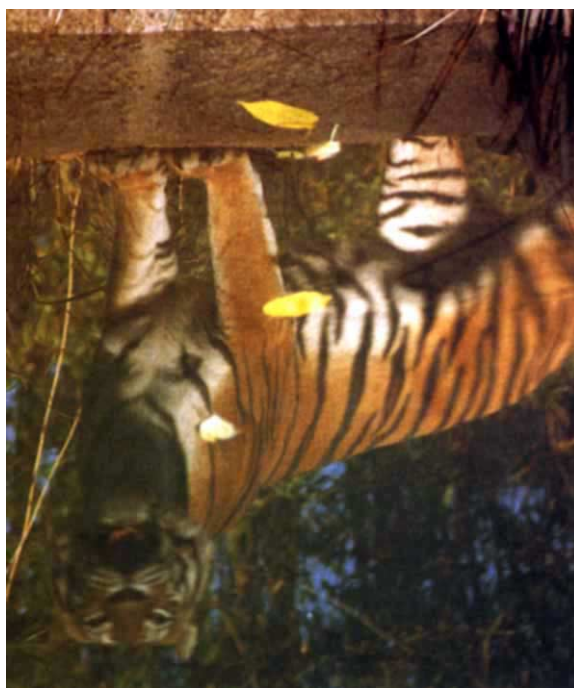
devoted to two unrelated tasks, and so the field has been in search of a unifying explanation.

Bradley *et al.* have now provided it. They recorded from neurons in area MT of rhesus monkeys trained to fixate on a display screen. Individual neurons were first stimulated by a pattern of dots

same visual plane, and that it becomes weaker as the disparity increases. Similar results are obtained whether the two patterns overlap or are merely adjacent. The results suggest a simple explanation for how and why MT integrates direction and depth cues. Transparent motion normally arises when objects pass each other in different planes. By not having inhibition between movement in different planes, the brain can interpret the two movements as independent. If instead the two movements occur within the same plane, transparent motion is less likely, and the brain looks for other explanations (for instance, random noise).

Is this consistent with what we know from human psychophysics? In fact, for simple patterns such as two sets of dots moving past each other, subjects have no difficulty in perceiving two sets of coherent motion, even when both occur in the same plane. But for more complex stimuli, the task becomes more difficult, and the authors previously showed that stereoscopic disparity between the two directions can improve performance. They suggest that, in the real world, the visual system makes use of many clues to arrive at the correct interpretation, and they raise the possibility that MT may exploit not only depth, but also other features such as colour or texture to distinguish between the components of transparent motion. Faced with threats such as tigers hiding in the undergrowth, it is easy to believe that the primate visual system will have evolved to use all the help it can get.

Charles Jennings



moving in their preferred direction. Having confirmed the inhibitory effect of adding further dots moving in the opposite direction, the authors separated the two sets of dots into different visual planes (with coloured glasses similar to those used for viewing old 3D movies). They found that the inhibitory effect is strongest when both sets of dots lie in the

matically increase the efficiency of information encoding. For example, if in a population of 1,000 ideal binary neurons, only one neuron discharges at any time, then that population can encode only 1,000 states, or about 10 bits of information. But if 5 per cent of the neurons are active at any time, the same population can encode as much as 282 bits (or 10^{85} states)³. Population coding, however, seems to require a mechanism by which all the neurons representing one thing are related or 'bound together'⁴⁻⁶. One theory is that neurons signal their relatedness by discharging nerve impulses in synchrony with each other at about 40 Hz. This is an attractive theory because 'brain waves' of various frequencies have been observed since the first electroencephalographic (EEG) measurements were made in humans in the 1920s, but the function of these brain waves has never been clear.

Quite simple feedback systems are prone to oscillation, so given their many recurrent connections it is not surprising that cortical circuits do the same. And it is exactly these recurrent circuits, which are similar to the attractor neural networks described by Hopfield⁷ and others, that may provide a basis for associative recall. Unfortunately, classical associative memories remember and recall entire input scenes as single memories. Subcomponents of the scene (such as the face of our friend in the crowd) cannot be recalled independently. This restriction severely limits the ability of the associative networks to generalize, because commonly occurring components of complex scenes will not be recognized in new contexts^{4,5}.

So the theoretical and experimental challenge is to find a method that enables an associative memory to segment a scene into a number of components in a composite input and represent them separately and simultaneously. Theoreticians have now demonstrated that if associative networks are allowed to oscillate, then this problem can be partly solved. These oscillatory networks are able to express a few memory patterns simultaneously by representing each pattern by an oscillation of a different phase⁵. Thus, the discharge of neurons representing the components of any one pattern becomes temporally correlated, but anticorrelated with the discharge of neurons representing all other patterns.

A key link between collective oscillations and perception was made by Gray and Singer (see ref. 6) when they noticed that cortical neurons in the visual system responded to coherent visual stimuli by discharging synchronously at frequencies of around 40 Hz. Incoherent stimuli comprised of unrelated features did not elicit synchronous discharge. This suggests that the synchronization reflects a transient binding together of reverberating groups of neurons, each of which responds to a

different feature of the same single perceptual object. Whittington *et al.*¹ have now been able to demonstrate that these 40 Hz oscillations can be evoked in thin slices of brain that were kept alive in a nutrient bath. The advantage of the brain slice is that neurons can be studied far more easily than in the whole brain. Whittington *et al.* were able to show pharmacologically that only a small subset of the known neurotransmitter receptors on hippocampal neurons were needed to generate the 40-Hz oscillatory activity of the network. The metabotropic subclass of the glutamate receptors, which are potent activators of the inhibitory neurons, were sufficient to provide tonic excitation, while the GABA_A subclass of inhibitory receptors provided sufficient inhibition to gate the excitation and generate the oscillation.

Theoretical studies of network oscillations have concentrated on the interaction of inhibitory neurons with excitatory neurons, and have often incorporated neurons with intrinsic oscillatory properties. The computer model of Whittington *et al.* is radically different. It consists of a network of inhibitory neurons, none of which is an intrinsic oscillator, driven only by tonic excitatory activation, yet it predicts their experimental results. In the context of hippocampal memory, their decision to explore the metabotropic glutamate receptor is delightfully perverse, because the NMDA receptor, another type of glutamate receptor, has long been the cynosure of theoreticians and experimentalists studying 'learning' synapses in the hippocampus.

Whittington and colleagues' complex experiments and simplifying theory have identified the synapses between the inhibitory neurons as being the critical components in determining both the occurrence of oscillatory activity and the frequency of the oscillations. The way is now open for direct studies of the details of the oscillatory mechanism, and its possible associative function, under the controlled conditions of the brain slice preparation. □

Rodney J. Douglas is in the Department of Biomedical Engineering, Imperial College, London SW7 2AZ, UK. Kevan A. C. Martin is in the MRC Anatomical Neuropharmacology Unit, Mansfield Road, Oxford OX1 3TH, UK.

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DAEDALUS

Carbonated metal

MANY inventors have tried to make a foamed metal. None has really succeeded. No known detergent will whip molten metal up into frothy foam. Even in zero gravity, such a foam 'breaks' rapidly by the merging of its bubbles. Now Daedalus has a new approach.

He points out that ordinary chalk, calcium carbonate, has nearly the same density as aluminium. Accordingly, if chalk dust were suspended in molten aluminium, it would stay in suspension, or at least would separate out only slowly. But at the temperature of molten aluminium, calcium carbonate is beginning to decompose into lime and carbon dioxide. It has an appreciable vapour pressure. If the hydrostatic pressure falls below that value, each chalk particle will blow a bubble of carbon dioxide and turn into a grain of lime. Furthermore, the heat absorbed by the reaction will freeze a thin skin of solid aluminium around the bubble, stopping it from coalescing with others nearby. The result: foamed aluminium.

Pure aluminium and pure chalk are not quite perfectly matched for this process. DREADCO's chemists are stirring mixtures of various metal carbonates into a wide range of aluminium alloys, seeking the most compatible combination of density, melting point, vapour pressure, and heats of reaction and solidification. The final optimized process will melt the alloy under high pressure, stir in the carbonate powder, and allow the homogenized mixture to escape through a nozzle into normal atmospheric pressure. The alloy will foam, expand and solidify. If the nozzle is too narrow to permit full expansion, the foam will elongate instead, deforming its internal bubbles into long parallel ellipsoids or cylinders. Either way, a continuous column of foamed metal will emerge from the outlet.

Foamed metal will be a splendid new material. Like foamed polystyrene, its cellular structure will make it wonderfully light and rigid. Its lime inclusions will strengthen it further by impeding plastic flow (some turbine alloys are loaded with ceramic grains for this reason). Yet when forcibly bent it will tolerate drastic deformations. In all fields of engineering it will displace fully dense metal. Shipbuilders in particular should welcome a metal that floats. The grade with elongated bubbles will have the grain and character of hardwood, and may be best worked by carpenters — although the gritty lime will rapidly blunt their drills and saws. It will be easily and firmly joined by nails and wood-screws, but not by the traditional fish glue.

David Jones

Decline of melanic moths

SIR — Industrial melanism has been best documented in *Biston betularia*, the peppered moth. Melanic variants, unknown before 1848, all but replaced paler forms by the turn of the century in populations near British industrial centres. Distant rural populations, however, changed little¹. The changes have been attributed to habitat modifications and differential predation on the moths by birds¹.

Conditions began to reverse following the creation of smokeless zones in the United Kingdom initiated by the Clean Air Act of 1956. Beginning in 1959 the *Biston* population at Caldy common, 18 km west of Liverpool, has been sampled each year². There the frequency of the melanic form has dropped from a high of 94.2% in 1960 to its current (1994) low of 18.7% (65 melanic of 348). Similar reversals are well documented elsewhere in Britain, but the Caldy Common sample is by far the largest, with nearly 18,000 specimens taken to date.

Melanism has also been reported in the American subspecies of the peppered moth, *B. betularia cognataria*. Melanics have remained at low frequencies in rural Massachusetts between 1958 and 1977 (ref. 3), and in the Virginia mountains (1968–94, D. A. West, personal communication); whereas in central Pennsylvania melanics were once appreciably high in frequency and fell from 52 to 38% between 1971 and 1986 (ref. 4). The only North American location where melanic *Biston* has been recorded at frequencies comparable to British samples (exceeding 90%) is in southern Michigan⁵. Between 1959 and 1961, a combined total of 576 *Biston* have been collected at the E. S. George Reserve, a rural field station near Detroit. The sample sizes ranged from 24 to 173 over five generations (in Michigan the species is bivoltine), with no statistical difference in the distributions of melanics to non-melanics ($G_4 = 1.433$, $P > 0.75$). In all, 515 of the 576 were melanic⁵.

To determine if the frequency of melanic *Biston* has changed since the last census was taken at that location more than 30 years ago, we ran moth traps there for 7 weeks during last summer. Because of comparable sample sizes and the sample intervals, the most appropriate comparison is between the last sample taken (second brood, 1961) and the current sample. That 1961 sample, with 22 melanics and 2 non-melanics ($n = 24$), and the 1994 sample of 4 melanics and 21 non-melanics ($n = 25$), differ very significantly ($G_1 = 31.99$, $P < 0.001$).

The 1994 Michigan and the 1994 Liverpool samples do not differ in the frequency of melanics ($G_1 = 0.115$, $P > 0.5$). Although there are no intermediate sample points from the George Reserve,

the nearly matching drop in the frequency of melanics from above 90% to below 20% at these two locations over the same 35-year time interval is either an extraordinary coincidence, or parallel evolution.

A "parallel" Clean Air Act in 1963, with subsequent amendments, also led to reductions in atmospheric pollution in the United States⁶. Despite general improvements in most indicators of air quality, southeastern Michigan falls within the acid-rain belt⁶. However, the George Reserve was not conspicuously blackened before clean air legislation, nor has the habitat changed in this regard since then (D.F.O., unpublished data). It has remained a woodland habitat of mixed deciduous trees, many of them dark-barked oaks (*Quercus* spp.) and black walnuts (*Juglans nigra*). Furthermore, there has been no perceptible change in the lichen flora in that region of Michigan over the past 30 years (H. Crum, personal communication); thus, it appears that the rise and subsequent fall in melanism in the *Biston* population on the George

Reserve is not related to lichen succession. Although the evolution of melanism in this southern Michigan population seems to have paralleled the changes in British populations in both directions, common causes for the changes are not obvious. Clearly, more extensive sampling of American *Biston* is needed.

Bruce Grant

Department of Biology,
College of William and Mary,
Williamsburg, Virginia 23187, USA

Denis F. Owen

School of Biological and
Molecular Sciences,
Oxford Brookes University,
Oxford OX3 0BP, UK

Cyril A. Clarke

Department of Genetics
and Microbiology,
University of Liverpool,
Liverpool L69 3BX, UK

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Hydrophobicity and phylogeny

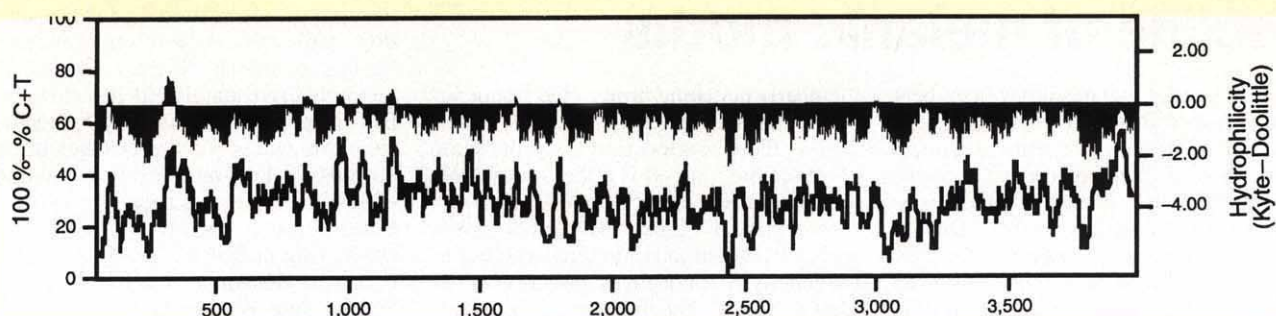
SIR — Mitochondrial DNA (mtDNA) sequences are routinely used to infer the pattern and timing of evolutionary divergences¹. Various methods for estimating phylogenies are used, each based on a different model of evolution. All these methods assume some model of evolutionary change, whether explicitly, as is the case for the likelihood procedures, or implicitly, as is the case for other methods^{2,3}. A lack of correspondence between an underlying model and actual evolutionary processes can lead to failure of a method. Unfortunately, several of the underlying models fail to adequately explain mtDNA sequence evolution^{4,5}. This suggests that factors beyond those accounted for by the models are acting on mtDNA sequences. Incorporating these additional influences into our models will enhance our ability to correctly recover phylogenies by improving the correspondence between the model of evolution and actual evolutionary processes. Here

we describe the mechanistic basis of a constraint acting on mtDNA sequences that represents one such influence.

It is well known that sites within DNA sequences differ in their relative freedom to vary⁶. In protein-encoding genes, third positions of codons typically vary more than first positions which, in turn, vary more than second positions, owing primarily to the differential degeneracy of the genetic code at these positions. It is often assumed that more rapidly evolving sites are more likely to result in chance matches (homoplasy) than are more slowly evolving sites when distant phylogenetic comparisons are made⁷. This may not necessarily be the case. If the 'character state space' (the number of possible states a site can exhibit) of a slowly evolving site is highly constrained, it could retain less phylogenetic information than a more rapidly evolving site for which more character states are available, because the probability that multiple substitutions will result in chance matches (homoplasy) across taxa increases as character-state space becomes more tightly constrained.

Constraints on character state space in DNA sequences are often reflected as deviations from a 1:1:1:1 ratio of the four bases A, G, C and T. This condition is termed base compositional bias. We have investigated compositional bias at all three codon positions for the 13 protein-encoding genes in each of 12 taxa (see figure caption). There does not seem to be a

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Correspondence between base composition at second positions of codons for the 13 human mitochondrial protein-encoding genes (lower histogram) and the hydrophilicity of corresponding amino acids calculated by the Kyte–Doolittle method (upper histogram) using the MacVector Program (IBI). Base compositions were computed by averaging C and T frequencies using a sliding window of 40 bases for the entire sequence. Similar figures and patterns of base compositional bias at the three codon positions of protein-encoding genes were also determined for rat, mouse, cow, possum, chicken, frog, carp, lancet, sea urchin (2 species) and *Drosophila* (data not shown).

strong bias for or against any of the four bases at the first position in these taxa. At second positions there is a striking compositional bias for C and T. Finally, there is a strong bias for A (or against G) at third positions. The different base compositions probably reflect different constraints operating at each of the codon positions.

We have found that the bias for T and C at second positions is directly correlated with hydrophobicity (see figure). Of the 26 triplets encoding hydrophobic residues, 24 (92%) have T or C at the second position. Mitochondrial proteins contain a high proportion of hydrophobic residues, that are frequently clumped together into domains. These domains probably represent membrane-spanning segments that must be hydrophobic to ensure their conformational stability^{8–10}. This functional requirement for hydrophobicity effectively constrains character-state space at second positions to one of two states, T or C, causing sec-

ond-position sites to cycle between these two states over evolutionary time. Ironically, these positions, hitherto regarded as perhaps the most reliable for inferring evolutionary histories of distantly related taxa, may actually carry less phylogenetic signal and be less reliable than the more fast-evolving first positions whose compositional bias is less skewed.

Here we have identified one constraint that influences mtDNA sequence evolution. If we are to build realistic models of DNA sequence evolution that better allow us to retrieve phylogenetic signal from such data, it will be important to identify others.

**Gavin J. P. Naylor*, Timothy M. Collins
Wesley M. Brown**
Department of Biological Sciences,
University of Michigan,
830 N. University Avenue,
Ann Arbor, Michigan 48109-1048, USA

* To whom correspondence should be addressed at: Department of Zoology, Arizona State University, Tempe, Arizona 85287-1501, USA.

Complex bacterial patterns

SIR — Budrene and Berg¹ have studied patterns of spots, stripes and rings formed by motile cells of *Escherichia coli* grown in a thin layer of semi-solid agar. Similar patterns are produced by *Salmonella typhimurium* (Y. Blat and M. Eisenbach, personal communication). Here we propose that the interplay of a few common cooperative strategies underlies this diverse set of phenomena. We use two kinds of models to demonstrate that these strategies (the generic features), rather than the details of the models, are crucial for producing the observed patterns.

The most familiar strategy for ensuring cooperative behaviour is attractive chemotaxis. Indeed, Budrene and Berg¹ proposed that a chemoattractant mediates the observed spot formation in *E. coli*. This is easily confirmed by using a model of growth that includes nutrient diffusion and consumption, and bacterial

growth and bacterial motion². The addition of a diffusing attractant *c* that is constantly emitted by the bacteria, together with bacterial motion towards its gradient, leads to the creation of spots. We show results of numerical simulation of a continuous model including these features (*a* in the figure).

This simple model is insufficient, however, to explain several crucial observations¹. In the experiments, spots appear sequentially in the wake of a spreading broad ring and later 'lock' into position as the bacteria turn non-motile. To capture these effects one must introduce additional mechanisms. First, we explicitly include in the model a 'triggering' field *w* (a field that must reach a threshold before attractant emission is activated). The value of this threshold may depend on the ambient chemoattractant concentration. In detail, if *c* > *c*₀, emission occurs when *w* > *w*₀ and otherwise, when

w > *w*₁ (where *w*₁ > *w*₀). Biologically, *w* represents the hazardous waste products produced by the bacteria. Also, bacteria in the full model differentiate into a non-motile state when starved for a sufficiently long time. To observe (in the simulations) different patterns, we vary the model's parameters related to the bacterial response to the triggering field and to the precise nature of the chemoattractant signalling. The final continuous model is then in good agreement with many of the features seen in experiment, including the possible radial organization of spots or stripes (*b* in the figure).

An alternative model — the 'communicating walkers' approach³ (introduced in the study of branching patterns during growth of *Bacillus subtilis*) — leads to the same results. The pattern shown in *c* of the figure was produced by the discrete model described in ref. 3 which we have modified to apply to *E. coli* in semi-solid agar. The 'freezing' of a walker (which stops moving after an interval of time during which not enough food is available) represents the observed differentiation into a non-motile state of the individual bacterium. Augmenting the bacterial motion and nutrient dynamics are the following additional features. First, a 'triggering' field (representing the waste products). The field is produced by the bacteria at a rate proportional to the rate of food consumption and is decomposed by the bacteria at a fixed rate. Second, a chemoattractant field. The emission of the chemoattractant is stimulated when the triggering field exceeds a threshold value or when the concentration of the attractant in the surrounding of the walkers exceeds a minimum value. Once a walker starts to produce attractant, it does so at a fixed rate for an interval of time such that a fixed amount is released.

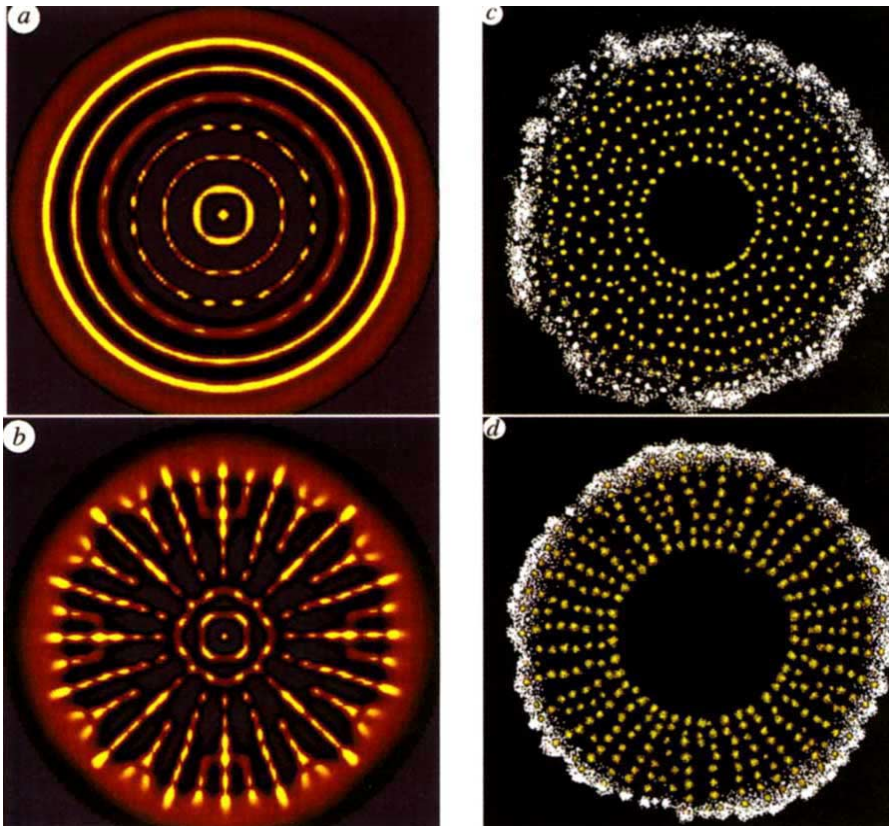
The walkers approach has its computational advantages and disadvantages relative to the continuum approach. As each walker represents a coarse-graining of about 10⁴–10⁵ bacteria (numerically, one cannot handle 10¹⁰ particles, which is the number of bacteria in a colony), it has an

Biological levels

SIR — As a chemical engineer who has worked in cellular biology, I read with interest your article “Gaps in Molecular Biology” in *Nature*’s 125th anniversary issue¹. From my perspective, I see a serious limitation in a fundamental guidepost underlying current work in molecular and cellular biology: that a satisfying understanding of complex biological processes will follow from a complete inventory of the molecular components and a knowledge of their structure and reactions (for example, Fig. 6 of ref. 2).

The notion that “knowing all the players” and “who talks to whom” (I. Dawid, cited in ref. 3) will lead to biological enlightenment involves a tacit assumption of linear reactions. Indeed, *Nature* and other journals commonly summarize advances in molecular biology with linear reaction sequences, for example, Ras activates Raf activates MAPK, and so on, or matrix sums of such sequences. But the vast majority of biological reactions are nonlinear (for example, $A + B \rightarrow C$, or $2A \rightarrow B$) and the cell appears as an open system far from equilibrium. In chemical engineering it is understood that as few as four nonlinear reactions in an open system can lead to a spectacular array of limit cycles, chaos, and multiple steady states (see ref. 4). Added complications such as diffusion and concentration gradients lead to unpredictable spatial patterns as well.

Thus one can know all the apparent players, the principal reactions and the rate constants, and yet be unable to predict a specific outcome. The cell is seen to have all the behaviours above and biologists routinely find that some small insults push the cell past a critical point and initiate distant effects, yet linear reasoning persists in most experimental approaches. For example, a common experimental strategy of determining molecular function is to increase or decrease a particular reactant, decreasing expression of a particular gene product, say, and then measuring the consequences. But in complex systems, there may be little or no effect from such interventions because chaos, for example, can provide unexpected stability. The evident surprise of molecular biologists on the organismal effects of many gene ‘knockout’ experiments may be based on a relative lack of appreciation of nonlinear effects in chemical systems. I find it ironic that the standard wall charts of biochemical reactions show hundreds of coupled nonlinear reactions, while graduate biology students are tacitly encouraged to think in terms of linear cause and effect.



Results of the numerical simulations of the two models. *a*, Spot formation in a continuous model where the bacteria continuously emit chemoattractant. The growth front breaks into rings which subsequently break into spots (colours indicate bacterial density, with purple the lowest density and yellow the highest). *b*, Radial spots pattern in a continuous model which includes the chemoattractant triggering field described in the text. The dynamics of growth consists of a wide outer band which expands, while the spots are created in its wake (same colour code as in *a*). *c*, Spot formation in the ‘walkers’ model. The spots are formed in the wake of a spreading circular band (yellow indicates non-motile walkers and white indicates motile walkers). *d*, Observation of a radial structure of spots when the chemorepellent mechanism is included in the walkers model (same colour code as in *c*). (Contact authors for details).

inherently high level of noise. However, it is more flexible in terms of the easier incorporation of complex bacterial response, such as a possible refractory period after emission of chemoattractant or finite emission time. For example, the pattern shown in *c* was produced in the presence of such a finite emission period. In general we found that ‘sunflower’-like patterns are formed more easily when finite emission period is included and for high levels of the waste threshold w_0 .

As we have seen, radially organized structures can be produced if the triggering mechanism is taken to be attractant-concentration-dependent. In the absence of this autocatalytic effect, neither model could produce the observed radial structures. Motivated by the proposed repulsive chemotaxis in *B. subtilis*³, we have also considered the possibility that a sunflower-like pattern is changed to a radial structure due to a chemorepellent that is emitted by starved bacteria. We used the walker model to test this possibility and

found that chemorepellent signalling can indeed be an alternative explanation. In *d* we show an example of a simulated radial arrangement of spots in the presence of chemorepellent signalling. Other observed patterns such as radial stripes can be obtained if we change the relative strength of the repellent and attractant effects. This result suggests that *E. coli* and *S. typhimurium* might also use a repellent-attractant chemotactic interplay in the cooperative formation of complex patterning. A more detailed comparison with the time evolution of the observed patterns will be needed to distinguish between these hypotheses.

**Eshel Ben-Jacob, Inon Cohen
Ofar Shochet**

*School of Physics and Astronomy,
Raymond & Beverly Sackler Faculty of
Exact Sciences,
Tel-Aviv University, Tel-Aviv 69978, Israel*

Igor Aranson

*Department of Physics,
Bar-Ilan University, Ramat Gan
52900, Israel*

Herbert Levine, Lev Tsimring
*Institute for Nonlinear Science,
University of California, San Diego,*

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In a related vein, I am somewhat surprised that biology has not found more useful concepts at the intermediate length scale. Engineering examples include the arch in architecture, which connects the componentry level of bricks with the overall outcome of cathedrals, and viscosity in fluid mechanics, which connects the componentry molecular interactions with the overall fluid responses to force. Without such intermediate concepts, one is in the position of trying to understand cathedrals by cataloguing the bricks. Several phenomena in biology such as the gel-sol transition accompanying animal cell crawling may not be

understood on the basis of individual molecules and their interactions, but may be understood in terms of an intermediate phenomenon, thixotropy for example.

Robert E. Buxbaum

Department of Chemical Engineering,
Michigan State University,
East Lansing,
Michigan 48824,
USA

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Was the solitaire of Réunion an ibis ?

SIR — The Mascarene islands were the home of an extinct family of birds, the Raphidae, which were large-sized, flightless Columbiformes. This family includes the Mauritius Dodo, *Raphus cucullatus* (Linné), and the Rodrigues solitaire, *Pezophaps solitaria* (Gmelin). These two forms are known by contemporaneous

downwards than the bill of the different species of that genus. Now in one of the most detailed descriptions of the Réunion solitaire, Dubois writes that “the beak is like that of a Woodcock but larger”^{3,4}, therefore it must have been relatively long and straight. This description of the bill does not match the description of the Raphidae-bill, but could correspond to a relatively short, straight ibis-bill (see figure).

The other external characteristics noted by the early travellers can also correspond to an ibis related to the sacred ibis, *T. aethiopicus*, or to the straw-necked ibis, *T. spinicollis*. According to the different authors the Réunion solitaire was white, or grey and white, or “white with the tips of the wings and tail black”^{3,4}. In the sacred ibis, the wing tips are black with metallic glints, and cover the tail,

which is white. In the straw-necked ibis the neck, underpart and tail are white, while the back and wings are glossy black. Moreover, Dubois writes: “The tail feathers resemble those of an ostrich”. Now in the sacred ibis, during the reproductive period, the ornamental feathers situated on the back and at the wing tips, filiform, downturned, and longer than the tail, look more or less similar to the tail feathers of an ostrich. The solitaire of Réunion had a long neck and long legs. Together with the shape of the bill, these last two characteristics correspond to a bird that is very different from the Mauritius Dodo. Finally, the only indication concerning its food, given by Feuille: “Their food is but worms and filth (*saleté*) taken on or in the soil”^{4,5} corresponds much better to an ibis than to Raphidae; the latter were mainly frugivorous⁶.

The Réunion solitaire was observed by explorers between 1613 and 1708, and the

species probably disappeared around 1710–15 (ref. 5). According to what is known for the dates of disappearance of the Mauritius Dodo and the dates of the first description of the Rodrigues solitaire, it is likely that most of the travellers who gave an account of the Réunion solitaire did not have the opportunity to see these two forms personally. Moreover, after the disappearance of the genuine Dodo, the names “todaersen” or “dodo” were used to designate a very different bird⁵.

The main objection concerning the attribution of the Réunion solitaire to a member of the ibis family is the question of flight. Among all the writers who mentioned the solitaire, Castleton and Bon-teko described it as unable to fly, but Dubois indicates some ability to fly when he writes: “This bird has recourse to running, as it flies but very little”. The other writers do not state precisely whether or not it could fly^{4,5}. Now, except for one characteristic, the skeletal remains of the Réunion ibis do not indicate a reduction of flying ability. Because several authors described it as very fat, it may have had a seasonal fat cycle, with a fat period when it was unable to fly, and a thin period when it could

In conclusion, we think that the solitaire described by the early travellers was not related either to the Mauritius Dodo, or to the Rodrigues solitaire, but was probably the extinct Réunion ibis. In this case we propose to designate it as *T. solitarius* (Sélys-Longchamps, 1848), new comb., of which *Borbonibis latipes* is a junior synonym.

Cécile Mourer-Chauviré

Centre de Paléontologie
stratigraphique et Paléoécologie
associé au CNRS (URA 11),
Université Claude
Bernard - Lyon 1,
27-43 Bvd du 11 Nov.,
69622 Villeurbanne Cedex,
France

Roger Bour

Muséum national d'Histoire naturelle,
Laboratoire des Reptiles
et Amphibiens,
25 rue Cuvier, 75005 Paris,
France

Sonia Ribes

Muséum d'Histoire naturelle,
1 rue Poivre, 97400 Saint-Denis,
La Réunion,
France

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Comparison between the fragment of mandible of the Réunion ibis, from the Marais de l'Ermitage, and the mandible of a recent *T. aethiopicus*, the sacred ibis. The description by Dubois of the beak of the Réunion solitaire, “like that of a Woodcock, but larger”, matches that of an ibis with a relatively short, straight bill, and is very different from the bill of the Raphidae, which is very strong, inflated at the end, with a sort of incurved, downturned hook^{3,7} (36% of original size).

descriptions and illustrations, by a few anatomical remains for the dodo, and by a large number of fossil remains for both species.

In Réunion there existed a bird called the solitaire, exclusively known by historical reports. Excavations recently carried out in Réunion did not reveal any remains of Raphidae, but remains of an extinct ibis, *Borbonibis latipes*^{1,2}, were found in three localities and were particularly abundant in the Marais de l'Ermitage. This species must have been relatively common; strangely, the early travellers never mentioned it, although they mentioned almost all the species that have subsequently been found in the different fossiliferous localities.

The osteological analysis of *Borbonibis* shows that it is closely related to the recent genus *Threskiornis*. However, a fragment of lower jaw, found in 1994, shows that its bill was much shorter and less curved

Facing the facts

Erika L. Rosenberg

Human Facial Expression: An Evolutionary View. By Alan J. Fridlund. *Academic Press*: 1994. Pp. 369. £45, \$59.

THIS major treatise on the evolution of facial behaviour presents the most radical and controversial view on the topic since Darwin's *The Expression of the Emotions in Man and Animals* (1872). Darwin did his best to disprove the idea of his predecessors that our facial expressions are wilful acts. He argued instead that emotions can be interpreted from patterns of facial movement and that this link between emotions and expressions is innate.

Fridlund has now applied the concepts of modern genetics and evolutionary theory to the study of facial behaviour. But in doing so he contrives to shake the credibility of a century's research and subvert the status quo. He attempts to undermine the plausibility of Darwin's account; argues that the face cannot express *any* internal state, let alone emotion; and portrays the face as a signalling system while precluding its role as a response system.

Fridlund begins by discussing the history of the study of the face, the movement of facial muscles and the evolution of facial displays. The rest of the book is mainly devoted to contrasting his "behavioural ecology view" of facial displays with the more traditional "emotions view", a term he uses to refer to all theories in which the face has a role in emotional expression. Fridlund contends, however, that our facial displays reveal nothing about how we feel but rather signal to others what we might do. (In a smile for example we should read not happiness but a readiness to affiliate.) Our facial displays are in this way social, always implying the presence of an audience, even when made in private.

Fridlund proposes several interesting ways in which these signs of intended action may develop, including instrumental learning, shaping, imitation and cooption and conventionalization of reflexes. One of the more intriguing mechanisms is facial analogy, as in "the face I make to you is the one I make to ice cream". In Fridlund's words, "the faces we make in the world of people and ideas. . . build by analogy upon our infantile intentions toward tastes, smells and sights". This contrasts with the earlier proposal that we are born with the blueprints for facial expression of emotions. (Fridlund does not say how we learn to associate sensory reactions with social stimuli.) Equally compelling is the brief discussion of the paralinguistic role of the face. And impor-

tantly, Fridlund emphasizes that facial displays "coevolve with others' responsivity to them". This inclination towards a social ecology — especially within the auspices of an evolutionary theory — is a fresh approach. Ironically, however, Fridlund says nothing about whether our brains

facial expression and emotion judgement. Russell suggests that the findings on the universal recognition of emotion from facial expression have been overstated. Here Fridlund defers to Russell, without addressing the published claims that Russell has misinterpreted the literature, misquoted people and erred in his calculations. And, like Russell, Fridlund plays down the large amount of research demonstrating that many of the muscular patterns that are well recognized cross-culturally also appear in various spontaneous emotional contexts.

Fridlund's portrayal of the 'emotions view' is simplistic — the idea that the face

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"Horror" from Charles le Brun's *Characters des passions* (c.1720).

may have evolved to interpret the face as a cue to the inner world of our fellow human beings.

Fridlund is at pains to point out that Darwin's theory of facial expression was nonadaptationist. Darwin indeed argued that movements once "serviceable in gratifying some desire, or in relieving some sensation, if often repeated, become so habitual that they are performed whether or not of service, whenever the same desire is felt", and he used this Lamarckian idea to explain how facial expressions become involuntary and innate. But this is hardly surprising: belief in the inheritance of acquired habits was once common. Nevertheless, it seems likely that Darwin saw natural selection as a more powerful mode of evolution. Dwelling on Darwin's failure to provide an explicitly adaptationist account and his neglect of Mendelian mechanisms (not well known at the time) detracts from his seminal contributions. Darwin drew people's attention to the face as a correlate of emotion and to the idea that emotions may have an innate basis. He provided the foundation for the study of the psychology and biology of emotion.

Fridlund misrepresents work on the facial expression of emotion and the psychology of emotion more generally. For instance, the book reprints J. A. Russell's recent review of the literature on

expresses distinct feelings is merely one component — although his monolithic perspective helps him to simplify his argument. And his interpretation of old references is skewed. For instance, he claims that Ekman and Friesen said in 1969 that emotional expressions deviating from the 'prototypes' are 'admixtures' of the basic expressions. Fridlund implies that these researchers were thus suggesting that non-prototypical expressions result from mixtures of prototypes. In fact they were describing only what happens when elements of more than one type of basic emotion are seen on the face at once. What is more, Ekman has directly rejected the admixture approach.

Fridlund credits the 'emotions view' as an important step in the history of the study of the faces, but sees it as no longer useful. He tries to undermine its scientific status by attributing mystical qualities to it, likening its proponents to the early physiognomists who judged personality from facial features: "Likewise, the emotions' researcher divined emotional state from the face apart from the context in which the facial behaviour was emitted and observed. This iconic view of faces was Darwin's legacy. It was finally to prove inadequate to reconcile facial behavior with contemporary knowledge about human and animal communication."

But are Fridlund's 'behavioural ecology view' and the diverse set of perspectives that he labels the 'emotions view' really in opposition? They might simply operate at different levels of organization. His view is in principle reconcilable with certain types of facial expression consistently appearing when people are in certain emotional states. Fridlund's critics have argued that the face can signal emotions that in turn imply intended actions, thereby allowing it to signal both states and actions. Fridlund argues that "this solution, though diplomatic, is problematic", primarily because he believes there are no adequate criteria for evalu-

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Photographic plate from Charles Darwin's *The Expression of the Emotions in Man and Animals* (1872).

ating emotions. But there are features of emotional response — the covariation among behavioural, experiential and physiological systems — that are too coherent and too organized to have arisen by chance, and there is measurable linkage among systems. Emotions themselves may be adaptations, with facial behaviour merely one part of a complex response package. These facial behaviours are indeed involved in communication, yet this does not preclude their role in the organized emotional response.

Fridlund fails to entertain the possibility that there is selection pressure on humans to predict other people's internal states. Understanding emotions from faces provides information about how people may be feeling, what they may be thinking and how they might act. The remarkable consistency in our judgement of facial expression of emotion attests to the presence of such adaptations. □

Erika L. Rosenberg is in the Department of Psychology, University of Delaware, 220 Wolf Hall, Newark, Delaware 19716, USA.

Evolving together

Brian Charlesworth

The Coevolutionary Process. By John N. Thompson. *University of Chicago Press*: 1994. Pp. 376. \$49, £39.25 (hbk); \$19.95, £15.95 (pbk).

EVOLUTIONARY change resulting from interactions between two or more species is a topic that has attracted the interest of evolutionary biologists since the publication of the *Origin of Species*. Indeed, much of the thinking in this field can be traced back to Darwin himself. For example, his famous discussion of the causes of evolutionary divergence between species, in Chapter 4 of the *Origin*, laid great emphasis on the selective advantage of being different from competitors. His book on the *Various Contrivances by which Orchids are Fertilised by Insects* documented the intricate and intimate relationships between the behaviour and structure of insect pollinators and the structure of the flowers that they pollinate. It was the first detailed study of mutualism in the context of the theory of natural selection. Darwin noted that the Madagascar orchid *Angraecum sesquipedale* had nectaries that were 11 inches long and proposed that its pollinator must be a moth with a correspondingly long proboscis. This prediction was confirmed 125 years later by Anders Nilsson's discovery that the orchid is pollinated by a moth with an extraordinarily long proboscis, *Panogena lingens*. This must be one of the longest delays in the verification of a prediction in the history of science.

The first chapter of John Thompson's *The Coevolutionary Process* makes it admirably clear how great a debt the field of coevolution owes to Darwin's work. This is probably the most ambitious attempt so far to provide a general survey of coevolution. It contains an impressive array of fascinating case studies and examples, which will be of great value to researchers, teachers and students in evolutionary biology. Topics such as the evolution of host-plant specialization by insect herbivores, parasitism and mutualism are all covered in detail. The strength of the book is much more in the details of natural history than in the conceptual framework that Thompson develops to interpret it. Much of this framework is descriptive rather than causal. He argues at some length for the "geographic mosaic theory of coevolution" according to which local populations of a given species often evolve independently to engage in different kinds of interactions with other species. Apparently diffuse interactions of a species with several other species may in this way result from one-to-one interactions that involve different partners in different places. A major thrust of the early

chapters is that species (especially parasites) are often highly specialized with respect to the nature of their ecological interactions with other species, yet there is little hard phylogenetic evidence that specialization represents an evolutionary dead-end, as has often been argued.

Not a single equation to describe an evolutionary or ecological model is presented in this book, in sharp contrast to most recent books dealing with evolutionary ecology. Many biologists will probably feel this to be their gain rather than loss. But modelling helps to clarify the nature of the processes underlying observed patterns. Neglect of the predictions of models, however oversimplified they are, tends to lead to the substitution of vague generalizations for genuine theorizing. This is particularly evident in Thompson's discussion of the evolution of specialization. Despite devoting more than a hundred pages to this topic, he gives no clear account of why two competing species should diverge with respect to characters that influence their mutual competitive ability, and only one relevant theoretical study is mentioned. Similarly, the extensive theoretical literature on the coevolution of predator and prey species is given only a brief mention.

This book contains an immense quantity of useful and thought-provoking information and certainly deserves to be read by all evolutionary biologists. But it would have been a more stimulating work if the author had tried harder to define a clear set of hypotheses about the mechanisms of coevolution and to test them against the data that he obviously knows so well. □

Brian Charlesworth is in the Department of Ecology and Evolution, University of Chicago, 1101 E. 57th Street, Chicago, Illinois 60637-1573, USA.

Physical ambition

Eörs Szathmáry

Physical Approaches to Biological Evolution. By Mikhail V. Volkenstein. Translated by Artavaz Beknazarov. *Springer*: 1994. Pp. 399. DM98, \$67, £42.50.

THE late M. V. Volkenstein trained as a physicist and moved into biology from the area of polymer science. He was apparently willing to get his hands dirty and probe into established parts of 'real' biology. On the basis of this book, it would seem that his adventure was not entirely successful.

He covers three main areas: 'classical' biological evolution, molecular evolution and so-called physical approaches to evolution. His evolutionary heroes are

Charles Darwin — a kind of Darwin, of course — Motoo Kimura, Stephen Jay Gould and Gabriel Dover. Although Volkenstein discusses adaptation by natural selection, he is more enthusiastic about punctualism, nonadaptationism and neutralism — the further from adaptation, the better. This contrasts surprisingly with his admiration of such people as Darwin and Manfred Eigen. He discusses the latter's quasispecies model of replicating molecules at length, for example; the snag is that this is emphatically a model of mutation-selection balance. And we are told: "The amazing capabilities of the human brain originally had no adaptive significance. They emerged as a subsidiary trait as a result of the adoption of upright posture and the development of speech." Now, there is a lot to be said in favour of the adaptive significance of both of these characteristics. But how could one imagine speech without abstract thinking? Language is as much for internal representation as for communication. Volkenstein also forgets that it is a lot easier to learn something than to invent or discover it in the first place: inventing and discovering usually demand much more mental power.

This distorted view of Darwinian theory means that the author says nothing about the work and thought of J. B. S. Haldane, W. D. Hamilton and John Maynard Smith. I cannot recommend this part of the book to anyone: physicists will get a highly biased and partly incorrect picture of evolution, and biologists will become dissatisfied by the distortions and frustrated by some heavy-going mathematics. The best sections deal with proteins, the neutral theory and the quasispecies model — indeed, these sections are really good (ignoring the mathematical difficulties that some biologists may encounter).

Volkenstein's main concern is the contribution of physics to biological evolution. His survey is partly disappointing. Chaos theory is described at some length, but its serious application to ecology or evolution goes unmentioned. Eigen's quasispecies theory and the hypothetical hypercycles (cyclical systems of molecular mutualists) are seen as cornerstones of the physical approach: "this theory will make it possible to pass over to the physical treatment of evolution at the levels of cells and organisms". How this could be achieved remains a mystery. The author overlooks the fact that Eigen's theories are largely molecular and chemical (and not strictly physical); that the quasispecies model is isomorphic to a classical population-genetics model of haploid, asexual genomes in mutation-selection balance; and that, contrary to initial expectations, the 'naked' (that is non-compartmentalized) hypercycles do not satisfy the criteria for the integration of molecular information dispersed in unlinked replicators.



MORE than 100 million land mines are spread across 62 countries, killing or injuring some 1,200 people every month, over a third of them women and children. The United Nations funds most of the world's mine-clearance programmes, at a cost of \$300–\$1,000 per mine (some antipersonnel mines are worth as little as \$3). The task is slow and labour-intensive, usually involving civilians recruited locally and trained specially. This picture of a mine clearer on the Thai–Cambodian border appears on the cover of *Clearing the Fields* edited by Kevin M. Cahill. The book arose from a symposium held last year that brought together a group of internationally recognized authorities to examine the global threat of these buried and forgotten weapons and to propose practical solutions from ethical, legal, economic, medical and military perspectives. BasicBooks/Council on Foreign Relations, \$25 (pbk).

Volkenstein's account is sometimes dangerously misleading. For example, we are told that the distinction between developmental constraints and stabilizing selection as possible causes for evolutionary stasis is of no real importance (quite the contrary — lack of this insight leads to confusion); that stabilizing selection is aptly described by the Red Queen metaphor (the metaphor in fact applies to coevolutionary dynamics); and that the Eigen theory is a convincing model of the transition from nonlife to life (it is not, since it does not describe the origin of replicators, membranes or metabolism). No school of thought about the origin of life other than that of the Eigen group is mentioned: in particular, the reader remains ignorant of Wächtershäuser's surface metabolism or Gánti's chemoton model (which is based on the idea of metabolizing and reproducing compartments). The chemoton model is even partly explainable in mathematical terms and so should certainly have deserved space in a book arguing for the usefulness of a physicochemical approach.

Another potential contribution of physics to evolution has been inspired by synergetics, a branch of the physics of dissipative systems; it is often mentioned throughout the book but defined (to the annoyance of an innocent reader) only on page 333, which is just a bit too late. Volkenstein outlines a phenomenological

characterization of speciation events in morphological space, using phase transitions as a metaphor. The sad fact is that no attempt is made to couple these ideas to the genetic models of speciation or to the existing literature on the genetics of stasis and punctuation. It will be interesting to see how recent work on adaptive dynamics (by Hans Metz and his colleagues, for example) relates to this phenomenological approach.

The style of the book is very eclectic: long poetic allusions and citations are intermingled with hard-nosed science. I have nothing against either, but the author could have confined the former to footnotes or appendices.

Physicists should be wary of this book: although they will be fascinated by the problems and understand the mathematics, they will come away with an unreliable picture of the science of evolution. Biologists are no better off, for many of them will lack the necessary background in physics, chemistry and mathematics. The translation is good, some lesser-known Russian literature is covered and there are several illuminating sections, but all in all the book generates more heat than light. Sadly, the author failed to do justice to the fields and fulfil his ambition. □

Eörs Szathmáry is in the Collegium Budapest, Szentháromság u.2, H-1014 Budapest, Hungary.

Matchmakers

Stuart Sutherland

Mental Leaps: Analogy in Creative Thought. By Keith J. Holyoak and Paul Thagard. MIT Press: 1995. Pp. 320. \$24.95, £19.95.

NOWADAYS, in a few limited domains, computers equal or excel human intellectual capacities. They play chess at the grandmaster level and in certain branches of medicine they diagnose more accurately than expert medical specialists. But they have had no common sense — until recently. The simulation of common sense has been made possible by two developments: greatly increased computer storage and machines that can process information in parallel, thus vastly reducing the time needed to retrieve a particular concept. *Mental Leaps* describes an attempt to simulate one aspect of common sense — the ability to find appropriate analogies.

To construct their computer program, Keith Holyoak and Paul Thagard had to carry out a careful analysis of the nature of analogies. One example they use may have contributed to President George Bush's decision to start the Gulf War: he compared Hitler's occupation of Austria (the source of the analogy) with Saddam's invasion of Kuwait (the target). From this analogy one can conclude that Saddam's aggression would escalate unless something was done to stop it. According to the authors, analogies are based on three constraints: similarity (Hitler and Saddam were evil, occupation is similar to invasion); structure (the elements of the source must be mapped in a one-to-one way to those of the target (Hitler=Saddam, occupation=invasion and Austria=Kuwait) and the relations between the two sets of elements must be the same); and purpose, for example to discover how to solve a problem by analogy with one already solved or to persuade others, for instance by using case studies in law.

The authors proceed to describe a computer program for recovering analogies which makes use of these constraints to narrow the search for the appropriate source given a particular target. A network of connected nodes is established, each of which represents a possible correspondence between a target concept and a source concept. The nodes have excitatory and inhibitory connections between them, representing the three constraints. For example, the node Hitler=Saddam would have excitatory connections with occupy=invasion whereas Hitler=Saddam would have inhibitory connections with Hitler=Ho Chi Minh on the grounds that only one member of the

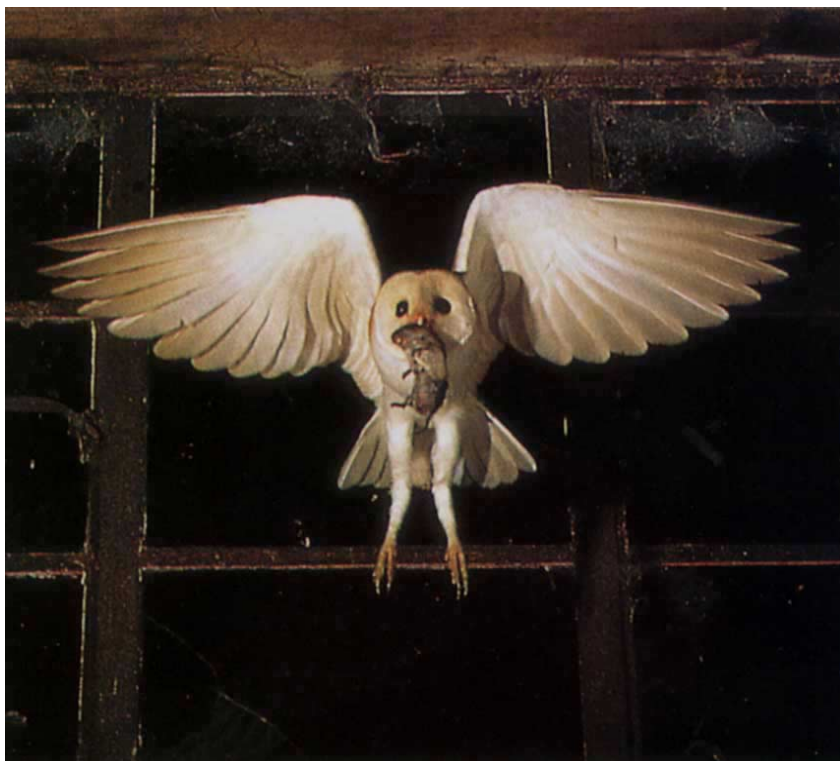
source can be matched to one member of the target. The strength of each node is changed iteratively according to the excitation and inhibition feeding into it from other nodes until there are no further changes in strengths, at which point there remains the most consistent set of nodes, those that still have high strength. These represent the analogy.

Three problems remain. First, it is easy to see how a clear-cut purpose can be used as a constraint in recovering an analogy. If we wish to show that Hitler is evil, it is no use matching him with Gandhi: the purpose provides a constraint. But it is not so clear how constraints would operate where we need a helpful scientific analogy, for example, atom=Sun and electrons=planets. All the relevant properties of the two systems would have to be set in one-to-one correspondence, but how do we decide what is relevant? Holyoak and Thagard are aware of the problem and emphasize that often the appropriateness of an analogy can be evaluated only through complex chains of reasoning after it has been found. Second, despite the parallel search used, it is not clear that the program avoids the combinatorial explosion. When tested on Aesop's fables as a source, the required number of iterations hardly increased from a choice

between fifty fables to a choice between a hundred. But the concepts in the fables would not have doubled — many of the second fifty would have appeared in the first fifty. Third, we are not told either how often the program failed to find an analogy or what proportion of the analogies found were appropriate as judged by people.

The book is nevertheless interesting and important, even if it sometimes makes heavy and perhaps unnecessary demands on its readers' application. In outlining what I regard as the most significant and original part of it, I have had to omit many subtleties. Moreover, most of it is devoted to a general discussion of analogies and their roles, presented in an attractive and novel way and covering among other topics the phylogenetic and ontogenetic development of analogical reasoning, the use of analogy in everyday life, politics, warfare, education and science, and the Chinese tea ceremony as an analogy for their way of life. To end with an avowal that the authors' program might or might not have recovered, I have been able to exhibit only a few pearls from the wares in the jeweller's shop. □

Stuart Sutherland is in the Laboratory of Experimental Psychology, University of Sussex, Brighton BN1 9QG, UK.



WITH its combination of ghostly pallor and eldritch shriek, the barn owl *Tyto alba* is perhaps the quintessential owl of popular imagination. It is captured here in its 'heraldic' pose, a symbol of excellence and sagacity (its prey is a short-tailed vole). From the informative and well-illustrated *The Barn Owl* by Mike Read and Jake Allsop. Blandford, £16.99.

Protein modules and signalling networks

Tony Pawson

Communication between cells assumes particular importance in multicellular organisms. The growth, migration and differentiation of cells in the embryo, and their organization into specific tissues, depend on signals transmitted from one cell to another. In the adult, cell signalling orchestrates normal cellular behaviour and responses to wounding and infection. The consequences of breakdowns in this signalling underlie cancer, diabetes and disorders of the immune and cardiovascular systems. Conserved protein domains that act as key regulatory participants in many of these different signalling pathways are highlighted.

CELLULAR interactions can be viewed as proceeding in two steps. Initially, an extracellular molecule binds to a specific receptor on a target cell, converting the dormant receptor to an active state. Subsequently, the receptor stimulates intracellular biochemical pathways leading to a cellular response, which may involve progression through the cell cycle and changes in cellular gene expression, cytoskeletal architecture, protein trafficking, adhesion, migration and metabolism. How are these internal pathways controlled and organized? Recently, a convergence of genetic, biochemical and structural data has focused attention on conserved protein modules that regulate signal transduction through their ability to mediate protein-protein interactions. These conserved modules represent common regulatory features of many distinct signalling pathways which are used to build up complex networks of interacting proteins.

SH2, SH3 and PH domains as building blocks

Many polypeptide hormones, as well as cytokines, antigens and components of the extracellular matrix, bind membrane-spanning receptors that signal through associated cytoplasmic protein kinase (PTK) domains. Although the targets of these PTKs may have quite different biochemical activities and biological functions, they often contain related sequences of 50–100 amino acids in length. These sequences, referred to as Src-homology-2 (SH2), Src-homology-3 (SH3) and pleckstrin homology (PH) domains^{1–3}, can each fold into a compact and functional module independently of surrounding sequences. SH2 and SH3 domains each recognize short peptide motifs bearing phosphotyrosine (pTyr) in the case of SH2, or one or more proline residues in the case of SH3. These conserved features are embedded in more variable sequences responsible for the high affinity and specificity with which individual SH2 or SH3 domains bind (Fig. 1). The binding properties of PH domains are less well understood, although they may associate with phospholipids or bind specific proteins. They may well promote the association of signalling proteins with membranes.

As they recognize pTyr, SH2 domains are explicitly involved in tyrosine kinase signalling pathways. Various combinations of SH3 and PH domains are frequently found in the same polypeptides as SH2 domains (Fig. 2), and can collaborate with them in forming signalling complexes downstream of PTKs. Indeed, proteins such as Grb2 and Crk are composed almost entirely of SH2 and SH3 domains, and apparently function as molecular adaptors to nucleate the formation of protein complexes, as discussed below. Polypeptides with multiple SH2, SH3 and PH domains can clearly engage in complex protein-protein interactions (Fig. 1a), which can regulate many facets of the signalling process, including enzyme-substrate recognition, enzymatic activity, and subcellular localization. The essential biochemical and structural properties of SH2, SH3 and PH domains are now described.

SH2 and PTB domains. Proteins with SH2 domains control biochemical pathways involving phospholipid metabolism, tyrosine

phosphorylation and dephosphorylation, activation of Ras-like GTPases, gene expression, protein trafficking and cytoskeletal architecture (Fig. 2)⁴. *In vivo*, SH2-containing proteins bind pTyr-containing sites on activated receptors and cytoplasmic phosphoproteins^{5–7}. These interactions can be mimicked *in vitro* using isolated SH2 domains and short phosphopeptides of 5–10 amino acids (Fig. 1b). SH2 domains bind to phosphopeptides of optimal sequence with relatively high affinity ($K_d = 10–100$ nM), and to phosphopeptides of random sequence with $\sim 1,000$ -fold lower affinity^{8–10}. They have essentially no affinity for unphosphorylated peptides. Most of the binding energy thus derives from pTyr recognition, although the amino acids surrounding the pTyr can increase the affinity by three orders of magnitude⁸. The phosphopeptide binding site is bipartite^{11–14}. A conserved pocket lined by basic residues binds the pTyr; this pocket contains the only invariant SH2 residue, an arginine which forms hydrogen bonds with two pTyr phosphate oxygens. The second binding surface is more variable, and allows specific recognition of the amino acids immediately C-terminal to the pTyr¹⁵. One group of SH2 domains, typified by those of Src and Lck, make specific contacts with the three residues immediately following the pTyr (the +1 to +3 residues; Fig. 1b). Such SH2 domains prefer hydrophilic amino acids at the +1 and +2 positions, but have a small hydrophobic pocket which accommodates a hydrophobic residue at the +3 position. The Src SH2 domain thus selects the sequence pTyr-Glu-Glu-Ile from a degenerate phosphopeptide library. By contrast, the SH2 domains of proteins such as the phospholipase PLC- γ 1 and the Syp/SH-PTP2 protein-tyrosine phosphatase (PTPase) recognize at least five, primarily hydrophobic residues following the pTyr, which fit into an extended hydrophobic groove running across the ligand-binding surface.

The bipartite organizations of the SH2 ligand-binding site allows tyrosine phosphorylation to function as an all-or-none switch for SH2 binding, while enabling the sequence context of the pTyr site to dictate which SH2 domains (and therefore which SH2 signalling proteins) are bound. Binding to pTyr sites can affect SH2-containing proteins in multiple ways, including direct stimulation of enzymatic activity¹⁶, relocalization within the cell¹⁷, and enhanced tyrosine phosphorylation¹⁸.

Recent evidence indicates that Shc and the insulin-receptor substrate IRS-1 may contain a novel pTyr-binding (PTB) domain of ~ 200 residues which bears only a very tentative relationship to SH2 sequences (refs 19, 20, 129; P. van der Geer *et al.*, manuscript submitted). The PTB domain appears to recognize motifs with the consensus Asn-Pro-X-pTyr, and to require residues N-terminal to pTyr for binding. Although the physiological significance of this domain is not yet known, it appears important for the interactions of Shc and IRS-1 with activated receptors (ref. 129; P. van der Geer *et al.*, submitted).

SH3 domains. SH3 domains are found in many proteins involved in tyrosine kinase signalling, but also in cytoskeletal components and subunits of the neutrophil cytochrome oxidase,

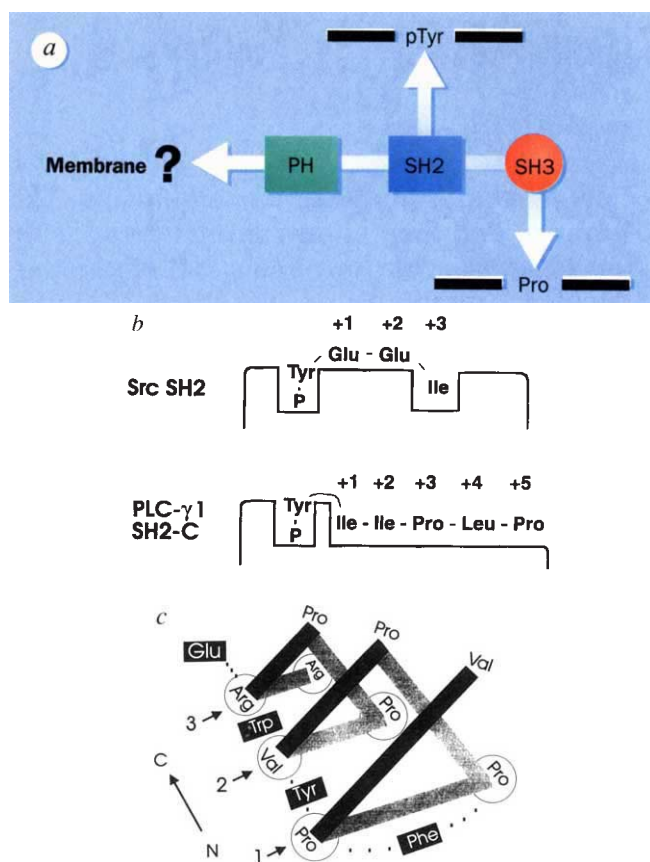


FIG. 1 SH2, SH3 and PH modules can form networks of interacting proteins. **a**, A protein containing SH2, SH3 and PH domains can potentially form multiple protein complexes. **b**, SH2 domains recognize pTyr-containing sites. Phosphorylation regulates binding, whereas the residues C-terminal to the tyrosine impart specificity. Peptides with the sequence pTyr-Glu-Glu-Ile bind with high affinity to the Src SH2 domain, which has a basic pTyr-binding pocket and a hydrophobic pocket for the +3Ile. The peptide pTyr-Ile-Ile-Pro-Leu-Pro-Asp binds the C-terminal PLC- γ 1 SH2 domain, which possesses an extended hydrophobic groove for the C-terminal residues. Structures for both these complexes have been solved^{11,13}, and are shown here in a highly schematic form. **c**, SH3 domains recognize proline-rich motifs. A proline-rich peptide derived from Sos is shown adopting a PPII helix as it binds the surface of a Sem-5/Drk/Grb2 SH3 domain in a class II orientation. Peptide residues that contact the SH3 domain are circled. Some of the SH3 residues that form the ligand-binding site are boxed. See text and refs 26–29 for details.

among others (Fig. 2)^{21,22}. SH3-binding sites consist of proline-rich peptides of approximately 10 amino acids (Fig. 1c)^{23,24}, which bind to isolated SH3 domains with dissociation constants of 5–100 μ M (ref. 25). Recent structural and mutagenic analysis of peptide-SH3 complexes^{26–30} shows that peptides associated with SH3 domains adopt a left-handed polyproline type II helix, with three residues per turn. The peptide ligand therefore has three spines, two contacting the SH3 domain, and the third stabilizing the PPII helix. The core ligand appears to be a seven-residue peptide containing the consensus X-P-p-X-P, where X tends to be an aliphatic residue and the two conserved prolines (P) are crucial for high affinity binding. The intervening scaffolding residue (p) also tends to be a proline. Each X-P pair fits into a hydrophobic pocket formed by conserved SH3 aromatic residues (sites 1 and 2), providing the principal binding energy. A third pocket (site 3) is more variable, although it frequently binds an arginine. In the peptide NH₂-R-A-L-P-P-L-P-R-Y-COOH, for example, which binds the Src SH3 domain, the first (underlined) arginine forms a salt bridge with an aspartate in the SH3 (ref. 26). The corresponding residue in the

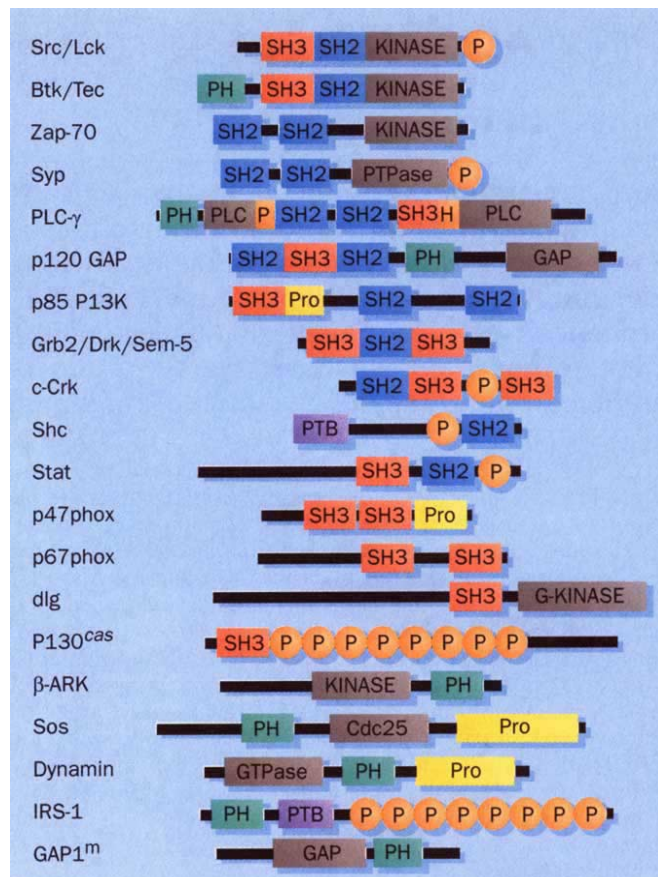


FIG. 2 Selected proteins (see text) with SH2, PTB, SH3 and PH domains. Pro, proline-rich SH3-binding sites; P, phosphotyrosine-containing SH2-binding site; PTPase, phosphotyrosine phosphatase domain; G.KINASE, guanylate-kinase-related domain; Cdc25, Ras guanine-nucleotide-exchange domain.

Abl SH3 domain is a threonine, which apparently dictates the selection of more hydrophobic residues such as methionine or tryptophan at this site in the target sequence^{28,30}.

SH3-binding peptides are pseudo-symmetrical and, remarkably, can potentially bind in either orientation (that is, amino- to carboxy-terminal as above (class I), or carboxy- to amino-terminal (class II)^{26,27,29}. In the case of a class II ligand for the Src SH3 domain, the arginine fitting into the site-3 pocket lies at the C-terminal end of the peptide, rather than at its N terminus. Hence the peptide NH₂-A-F-A-P-P-L-P-R-R-COOH binds with reasonable affinity to the Src SH3 domain, but in a class II orientation²⁶. The Ras guanine-nucleotide-exchange factors mSos1 and mSos2 are striking examples of physiological class II ligands. Sos peptides bound to an SH3 domain of Grb2, or its invertebrate homologue Sem-5, are complexed in the C- to N-terminal orientation, with an arginine forming a salt bridge to a glutamate in site 3 of the SH3 domain^{27,29}. In Sos SH3-binding sites the C-terminal arginine is usually followed by a further run of arginine residues, increasing the affinity for the Grb2 SH3 domains²⁶. The capacity of SH3 domains to bind ligands in either orientation both expands the range of potential binding partners, and has fascinating biological implications. For example, the ligand orientation will determine the spatial organization of the resulting complex, which may be critical for signalling.

Studies using degenerate peptide libraries and phage display libraries have indicated that each SH3 domain has a distinct binding preference^{24,25,30}. Specificity is apparently conferred by the interactions between non-proline residues in the ligand and two variable SH3 loops flanking the main hydrophobic binding surface.

There is no evidence that SH3-binding depends on a modification like phosphorylation, but the conformation of the SH3-containing protein or its binding partner may control the accessibility of the SH3 domain or its binding site. Potential binding partners may also be spatially separated, only interacting when juxtaposed by relocation. In addition, several SH3-binding sites have consensus sites for phosphorylation by proline-directed kinases, such as MAP kinase, which may affect the contacts made with the SH3 domain³¹.

PH domains—new kids on the block. PH domains have been found in a wide range of proteins, including serine/threonine- and tyrosine-specific protein kinases, substrates for these kinases, phospholipase C isoforms, regulators of small GTPases, the GTPase dynamin, and cytoskeletal proteins^{32–34} (Fig. 2). They appear to have a common structure, comprised of two roughly perpendicular anti-parallel β -sheets, followed by a C-terminal amphipathic α -helix³⁵. The loops connecting the β -strands are highly variable in length and sequence, and lie at the opposite face of the domain from the α -helix. The nature of PH domain ligands has been contentious. Phospholipids, notably phosphatidylinositol-4,5-P₂, have been reported to associate with the positively charged 'variable loop' face of the PH domain³⁶. X-ray crystallographic structures of the dynamin PH domain, however, reveal no extended hydrophobic cavity that could accommodate lipid side chains^{37,38}. PH domains have also been reported to bind the β/γ subunits of heterotrimeric G proteins through their α -helix and extended C-terminal sequences³⁹, as well as protein kinase C⁴⁰. The true nature of their physiological ligands, be they phospholipids, proteins, or both, remains to be established.

Several lines of circumstantial evidence suggest that PH domains may tether signalling proteins to membranes, a property consistent with the candidate ligands discussed above. First, PH-containing proteins are generally cytoplasmic, but associate with the plasma membrane. Indeed, cytoplasmic PTKs with N-terminal PH domains like Btk, lack the myristylated N terminus that anchors Src and its relatives to the membrane (Fig. 2). Similarly, the C-terminal region of β -adrenergic receptor kinase, which includes a PH domain, is crucial for recruiting the kinase to its membrane-bound substrate⁴¹. In the closely related rhodopsin kinase, the PH domain is replaced by a C-terminal farnesylation site. The main substrate for the insulin receptor, IRS-1, which acts as an intermediate SH2-docking protein in insulin signalling, also contains a PH domain which may play a role in juxtaposing the kinase and its substrate at the membrane. Although the PH domain is not essential for mitogenic signalling on insulin stimulation, it enhances IRS-1 phosphorylation in cells expressing low levels of the receptor, a property consistent with membrane localization (M. G. Myers and M. White, personal communication). The best evidence for PH domain function, however, comes from a genetic source. A missense mutation in the X-linked *btk* gene that alters an amino acid in the Btk PH domain impairs murine B-cell development⁴².

Receptors coupled through pTyr–SH2

Receptor tyrosine kinases. In many receptors, the extracellular ligand-binding domain and the cytoplasmic tyrosine kinase domain are part of the one polypeptide. Binding of the appropriate growth factor is accompanied by receptor dimerization and autophosphorylation⁴³. In the receptors for epidermal growth factor and platelet-derived growth factor (EGFR and PDGFR, respectively), autophosphorylation sites generally lie in non-catalytic regions of the cytoplasmic domain, between the membrane and kinase domain, in a non-catalytic sequence within the kinase domain, or in the C-terminal tail. A principal function of these sites is to bind the SH2 domains of specific receptor targets⁵, stimulating their activities. The β -PDGFR has multiple autophosphorylation sites, each of which is relatively specific for a particular SH2 protein⁷ (Fig. 3a). Mutant receptors lacking one or more autophosphorylation sites can therefore be used to examine the effects of uncoupling the receptor from specific

signalling pathways. Phosphorylation of Tyr 1,021 in the receptor tail, for example, induces binding of PLC- γ 1; however a mutant receptor in which Tyr 1,021 is replaced by Phe cannot bind PLC- γ 1 or stimulate hydrolysis of phosphatidylinositol-4,5-P₂, although it interacts normally with other SH2 signalling proteins^{44–46}. These and similar experiments have suggested that multiple receptor targets, including phosphatidylinositol-3-OH kinase (PI3K), Src and PLC- γ 1, contribute to efficient mitogenic signalling^{47,48}. Some of the affected targets may be functionally redundant, especially at the level of Ras activation⁴⁹.

Several receptor PTKs have similar features, although there are variations on the theme, and not all receptor SH2-binding sites show the striking specificity of the β -PDGFR. The receptor for hepatocyte growth factor, Met, has two closely spaced autophosphorylation sites, each of which binds multiple SH2-containing proteins⁵⁰. Substitution of these two sites abolishes Met mitogenic activity. By contrast, the main insulin-receptor substrate, IRS-1, has PH and PTB domains but is devoid of SH2 sequences⁵¹. It does, however, contain at least 20 potential tyrosine phosphorylation sites which, when occupied, form complexes with the SH2 domains of signalling proteins such as PI3K, Grb2, Syp and Nck⁵², and may therefore act as an intermediate SH2-docking protein, with more potential SH2-binding sites than can be accommodated within the receptor itself.

Multisubunit receptors. Antigen receptors in lymphoid cells employ similar interactions and signalling pathways, but with the ligand-binding chains, the tyrosine kinase catalytic activity, and the tyrosine-phosphorylated SH2-docking sites on separate polypeptides (Fig. 3b). T-cell activation involves a series of interactions between the antigen-presenting cell and the T cell. The polymorphic α/β chains of the T-cell antigen receptor (TCR) recognize specific antigenic peptides associated with major histocompatibility complex (MHC) proteins on the surface of the antigen-presenting cell. The MHC molecule associates with CD4 or CD8 proteins spanning the T-cell membrane, which bind the Src-family kinase, Lck, in the T-cell cytoplasm. The TCR itself forms non-covalent interactions with a series of transmembrane signalling subunits (γ , δ , ϵ and ζ), each of which contains one to three copies of the motif Tyr-X-X-Leu/Ile-X₆-Tyr-X-X-L/Ile⁵³. Phosphorylation of the tyrosines within these motifs in antigen-stimulated T cells (probably by Lck⁵⁴) creates binding sites for the SH2 domains of downstream-signalling proteins, notably another tyrosine kinase termed Zap-70⁵⁴. Zap-70 has two adjacent SH2 domains at its N terminus that may engage the closely spaced pTyr residues in each motif⁵⁵, activating the Zap-70 kinase domain. This in turn stimulates PLC- γ 1, leading to a rise in intracellular calcium.

The central role of Zap-70 in this pathway has been confirmed by a rare human immunodeficiency, in which CD8⁺ cells are not produced, and the remaining CD4⁺ peripheral T-cells do not produce IL-2 in response to antigenic stimulation. Without bone marrow transplantation, patients succumb to opportunistic infections at an early age. All patients studied so far have mutations in both *zap-70* alleles, disrupting the coding sequence for the kinase domain and preventing the production of detectable Zap-70 protein^{56,57}.

Although stimulation of this TCR signalling pathway is essential for T-cell activation, it cannot alone induce IL-2 production in quiescent T-lymphocytes. The necessary second signal can be delivered by the association of B-7, an immunoglobulin-like molecule on the surface of the antigen-presenting cell, and CD28, a transmembrane protein on T cells. CD28 has a short cytoplasmic tail containing the motif Tyr-Met-Asn-Met. Recent evidence indicates that the association of B-7 and CD28 induces the phosphorylation of CD28 at this site, allowing it to associate with the SH2 domain(s) of PI3K^{58,59}. Substitution of this Tyr with Phe abolishes CD28's ability to synergize with the T-cell receptor in stimulating IL-2 expression, indicating that its association with PI3K or other SH2 proteins is biologically important.

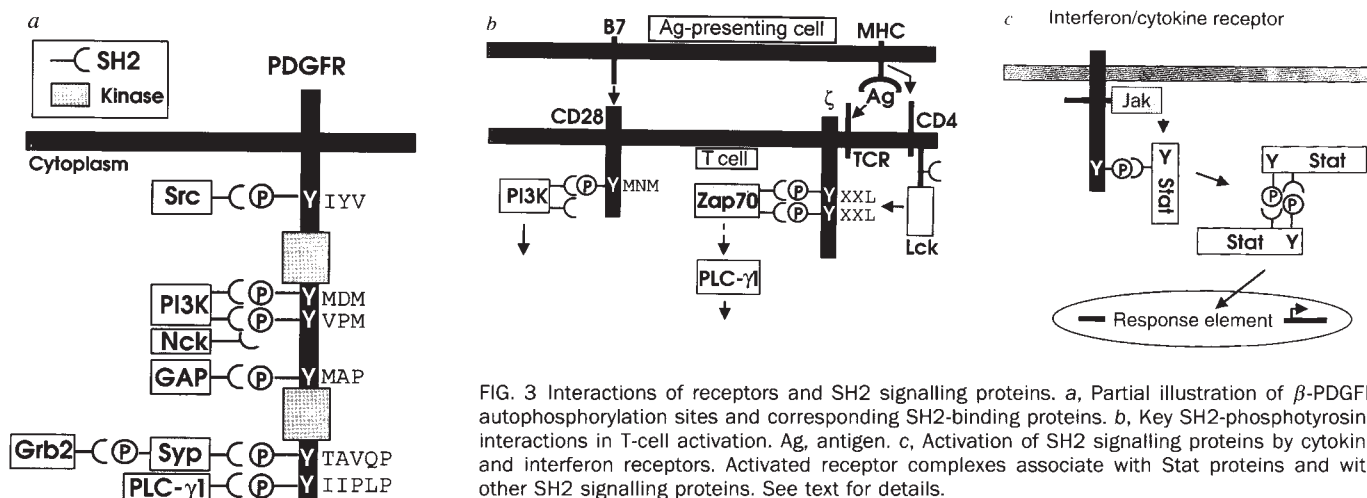


FIG. 3 Interactions of receptors and SH2 signalling proteins. a, Partial illustration of β -PDGFR autophosphorylation sites and corresponding SH2-binding proteins. b, Key SH2-phosphotyrosine interactions in T-cell activation. Ag, antigen. c, Activation of SH2 signalling proteins by cytokine and interferon receptors. Activated receptor complexes associate with Stat proteins and with other SH2 signalling proteins. See text for details.

In the absence of CD28, TCR stimulation induces a non-responsive state termed anergy, rather than activation.

In T cells, the activation of SH2 proteins therefore involves multiple subunits, and requires several discrete contacts with the antigen-presenting cell, each activating a distinct subset of SH2-regulated signalling pathways. This may represent a fail-safe mechanism for preventing the inappropriate activation of T-cells.

Cytokine receptors. The receptors for cytokines and interferons are composed of one to three polypeptides and are constitutively associated with members of the Janus protein kinase (Jak) family of intracellular PTKs (although the amount of associated Jak may increase on ligand binding^{60,64}).

Ligand-binding induces receptor oligomerization and concomitant Jak activation, possibly by cross-phosphorylation. The resulting tyrosine phosphorylation of the receptor signalling subunits enables them to bind SH2-containing proteins such as PLC- γ 1, PI3K, Shc, Syp and PTP-1C, which are in turn phosphorylated by the receptor complex^{65,66}. The PTK substrates most directly implicated in cytokine signalling, however, are transcription factors termed Stat proteins^{67,68}. These polypeptides possess an SH2 domain, through which they bind specific pTyr sites on the activated receptor. Each subfamily of cytokine receptors regulates a unique set of Stat proteins in a fashion that is determined by the SH2-binding specificity of the receptor phosphorylation sites¹³⁰. Subsequent phosphorylation of the Stat proteins at a tyrosine immediately C-terminal to the SH2 domain⁶⁹ induces their dimerization through mutual pTyr-SH2 interactions⁷⁰ (Fig. 3c). As a consequence, they translocate to the nucleus, bind to specific promoter elements and induce gene expression⁶⁰. Stat phosphorylation, translocation, and DNA-binding activity are all dependent on the invariant SH2 arginine residue. The location of the Stat pTyr site precludes an interaction with its own SH2 domain of the sort proposed for Src family kinases and Crk (see below), but permits dimerization by recognition of a second Stat molecule.

Pathways and networks

The Ras pathway. If proteins with SH2 and SH3 domains indeed mediate intracellular signalling by PTKs, then mutations affecting the ability of these domains to bind pTyr or proline-rich sites should block cellular responses to external cues. This hypothesis is supported by studies of Sem-5/Drk/Grb2, an adaptor protein that is involved in linking tyrosine kinases to Ras. Sem-5 contains an SH2 domain flanked by two SH3 domains, and couples the Let-23 receptor PTK to Ras in the differentiation of vulval progenitor cells in *Caenorhabditis elegans*⁷¹. Mutations affecting residues in the SH2 or either SH3 domain can uncouple Let-23 from the Ras pathway to a greater or lesser extent. In *Drosophila*, the homologue of Sem-5, Drk,

is required for signalling from multiple receptor PTKs, including the *Drosophila* EGFR homologue, and the torso and sevenless receptors^{72,73} (Fig. 4a). Flies homozygous for loss-of-function mutations in *drk* which alter basic residues forming part of the SH2 pTyr-binding pocket, die as pupae. Unlike wild-type Drk, these mutant Drk proteins neither bind to activated receptors nor become associated with the plasma membrane. Genetic and biochemical data indicate that Drk may act through Sos, a Ras guanine-nucleotide-release protein (GNRP) containing proline-rich motifs within its C-terminal tail, which bind the Drk SH3 domains. Mutations which inhibit binding of the N-terminal Drk SH3 domain to Sos block receptor signalling *in vivo* (T. Raabe *et al.*, submitted). The binding of SH2 and SH3 domains to pTyr sites and proline-rich motifs, respectively, is thus indeed critical for cellular responses mediated by receptor PTKs.

The mammalian homologue of Sem-5 and Drk is Grb2, whose SH3 domains bind mSos1 and mSos2, two mammalian homologues of *Drosophila* Sos⁷⁴⁻⁷⁸. Stimulation with growth factors such as EGF induces the Grb SH2 domain to bind motifs with the consensus sequence pTyr-X-Asn-X. Such a site lies at Tyr 1068 of the EGFR^{15,79}, recruiting Grb2 and Sos into a heterotrimeric complex with the EGFR and potentially stimulating Sos proteins to activate Ras. In metazoans, GTP-bound Ras activates a protein-serine/threonine kinase cascade, eventually triggering phosphorylation of transcription factors by MAP kinase⁸².

Many routes to Ras? This basic scheme, often depicted as a linear pathway from activated receptors to Ras (Fig. 4a), masks a number of complexities. Although the PDGF- and EGF-receptors can bind Grb2 directly^{79,83}, they also bind and phosphorylate SH2-containing proteins such as Shc and the Syp/SH-PTP2 tyrosine phosphatase, which contain Tyr-X-Asn-X motifs. *In vivo*, the Grb2 SH2 domain binds both to activated receptors, and to phosphorylated Shc and Syp^{84,85}, allowing Grb2 to form multiple complexes with other SH2-containing proteins as well as activated receptors. Biochemical and genetic data indicate that both the Shc-Grb2 and Syp-Grb2 complexes may stimulate the Ras pathway^{7,84,86}. Taken together, these observations point to a complex network of interactions synchronizing Ras activation with other signalling events (Fig. 4b). The network may also potentiate activation of the Ras pathway, functioning as a quantitative monitor of receptor activation. In addition, it enables PTKs lacking intrinsic Grb2-binding sites to activate the Ras pathway.

Like its SH2 domain, the SH3 domains of Grb2 can interact with several proteins *in vivo*. As well as mSos1 and 2, these include dynamin, a GTPase that may link Ras signalling to clathrin-mediated endocytosis^{87,89}. To add further complexity, mammalian cells contain several Ras GNRP apart from mSos1

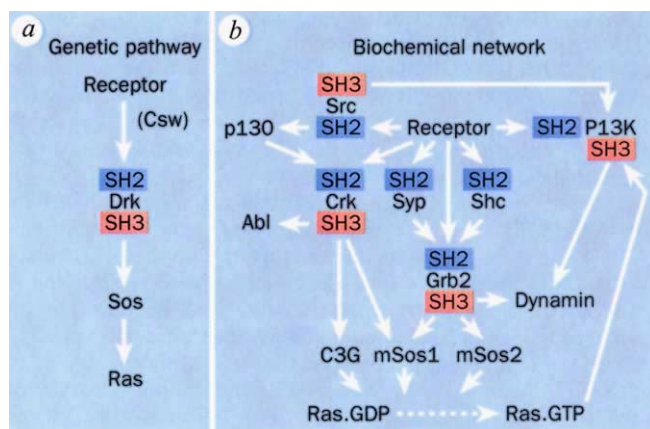


FIG. 4 Signalling through the Ras pathway. A linear pathway or more complex network? *a*, Genetic and biochemical experiments in *Drosophila* suggest a linear pathway, in which receptor PTKs couple to Drk (the homologue of Grb2), possibly with the SH2-containing PTPase Corkscrew (Csw; the homologue of mammalian Syp) acting as an intermediate¹²⁵. Drk then activates Sos. *b*, In mammalian cells, a complex network of interactions has been identified, involving proteins that can regulate Ras, only part of which is shown here. Interactions are as discussed in the text. See Fig. 2 for details of protein structures. The C-terminal proline-rich region of dynamin can bind not only Grb2 and PI3K, but also PLC- γ 1. The Crk SH3 domain interacts *in vivo* with both the Ras GNRP C3G, and also with a proline-rich motif in the C-terminal tail of the Abl PTK^{126,127}. PI3K makes multiple interactions, with receptor autophosphorylation sites through its SH2 domain, with the SH3 domain of Src family kinases through a proline-rich region of p85, and with GTP-bound Ras¹²⁸. These complexes, along with p85 tyrosine phosphorylation, may act synergistically to regulate PI3K activity.

and mSos2. One of these, C3G, has a C-terminal domain related to other Ras GNRPs, and can activate Ras in yeast⁹⁰. Its central proline-rich sequence binds the N-terminal SH3 domain of the adaptor protein Crk^{90,91}, and Crk-C3G complexes may thus, like Grb2-Sos complexes, couple tyrosine phosphorylation to Ras activation. Crk can also bind mSos1 *in vivo*⁹¹. Crk was first identified as a retroviral oncogene², and its transforming ability may be partly due to its interactions with C3G. The binding specificity of the Crk SH2 domain differs from that of Grb2 (pTyr-Asp-His-Pro), and may therefore expand the repertoire of tyrosine phosphorylation sites that can activate the Ras pathway (Fig. 4*b*). Engaging both the Crk and Grb2 SH2 domains may even enhance the extent of Ras activation.

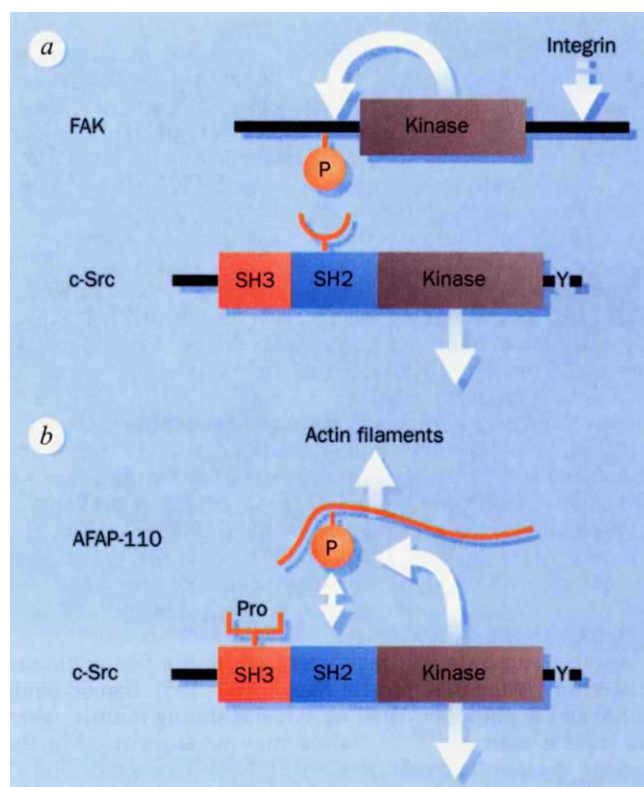
Finally, the mechanism of Sos activation may be complex, since changes in its catalytic activity have been hard to detect. One possibility is that Drk/Grb2 simply positions Sos in the membrane next to Ras, and it is clear that Sos membrane association is critical^{80,81}. However, recent genetic data in *Drosophila* have raised the possibility of a Drk-independent pathway to Sos (V. Banerjee, personal communication; T. Raabe *et al.*, submitted). Furthermore the N-terminal Sos PH

domain seems important for activation, perhaps through interactions with membrane components (M. Czech, personal communication), while the C-terminal Sos tail has an inhibitory function (ref. 81; S. Egan, personal communication). Drk/Grb2 may therefore activate Sos, at least in part, by relieving this repression.

Interconnecting pathways. Just as the exchange of GDP for GTP on Ras is controlled by multiple exchange factors, Ras inactivation is also regulated. Three mammalian proteins have been found to inactivate Ras (p120 GTPase-activating protein (GAP), neurofibromin and GAPI^m, collectively termed rasGAPs^{92,93}) by greatly enhancing the rate at which the GTP bound to activated Ras is hydrolysed to GDP. Enhancing or suppressing the activities of these rasGAPs can therefore affect Ras activation. Mutations in the gene encoding neurofibromin, for example, can in some cases elevate levels of Ras·GTP, thereby inducing aberrant proliferation. In a more physiological setting, transient inhibition of rasGAP activity may increase the amount of Ras·GTP in activated T-cells⁹⁴.

Two GAP proteins are directly implicated in the control of Ras by tyrosine kinases. GAPI^m has a PH domain, and is struc-

FIG. 5 Src family SH2 and SH3 domains have multiple functions. *a*, The Src SH2 domain binds other PTKs. In this scheme, tyrosine phosphorylation of Fak induces binding of the Src SH2 domain. This may stimulate Src activity by disrupting the interaction of the SH2 domain with pTyr 527 in the Src tail. *b*, The Src SH3 domain binds substrates. The SH3 domain of activated Src recognizes AFAP-110, which is then phosphorylated on tyrosine and associates with the SH2 domain.



turally similar to a *Drosophila* protein that functions as a negative regulator of Ras in the sevenless signalling pathway⁹³. p120 GAP possesses SH2, SH3 and PH domains, and binds to pTyr-containing proteins, including the β -PDGFR. A phosphoprotein (p190) that recognizes the N-terminal SH2/SH3 region of p120 GAP in growth-factor-stimulated cells is itself a GAP for the Rho GTPases involved in organizing the actin cytoskeleton^{95,97}. Control of gene expression by the Ras pathway and control of cell shape and adhesion by the Rho pathway may therefore be linked and coordinated through the association of the respective GAPs.

Src SH2 and SH3 domains

The Ras pathway demonstrates that SH2 and SH3 domains not only work in concert, but can also have multiple binding partners (and hence several discrete functions). Similarly, the SH2 and SH3 domains of the cytoplasmic PTK Src regulate kinase activity, bind substrates both before and after phosphorylation by the kinase domain, and direct subcellular localization.

SH2 and SH3 as regulators of Src. Src (like Lck and other related proteins) has an SH2 and an SH3 domain adjacent to its kinase domain. Kinase activity is repressed when Tyr 527, within the short C-terminal tail, is phosphorylated by Csk, another intracellular kinase, as long as both the SH2 and SH3 domains are intact⁹⁸. Phosphorylation at Tyr 527 thus appears to create a binding site for the Src SH2 domain, an event that also allows the SH3 domain to contact another part of Src. Together, these interactions prevent the SH2 and SH3 domains from binding to heterologous cellular proteins and lock the kinase domain in an inactive conformation. These interactions are generally thought to be intramolecular, largely because Src appears to be monomeric. Biophysical evidence indicates that an intramolecular SH2-pTyr interaction occurs in the Crk adaptor protein⁹⁹, suggesting that the same is true of Src. But the structure of the Lck SH2 and SH3 domains complexed with a phosphopeptide resembling the C-terminal Lck tail indicates that the inactive form of Lck (and by extension, Src) may be a dimer in which the SH2 and SH3 domains of opposing subunits are associated¹⁰⁰. Irrespective of whether their interactions are inter- or intramolecular, however, the Src SH2 and SH3 domains clearly have an autoinhibitory role.

Two mechanisms can thus be envisaged for Src activation: either dephosphorylation of Tyr 527 destabilizes the complex by releasing the SH2 and SH3 domains and activating the kinase domain, or high-affinity ligands for the SH2 or SH3 domains compete for the autoinhibitory interactions, with the same effect. For example, autophosphorylation of the β -PDGFR in the juxtamembrane region creates a high-affinity binding site for the SH2 domains of Src and its relatives Fyn and Yes, whose kinase activities are stimulated on association with the receptor^{101,102}. Similarly, autophosphorylation of the focal adhesion kinase FAK, a PTK activated by the adhesion of cell-surface integrins to extracellular fibronectin, creates a binding site for the Src SH2 domain¹⁰³, linking cell adhesion to the phosphorylation of Src substrates (Fig. 5a).

Src SH2 and SH3 domains in substrate recognition. Once Src is activated, its SH3 domain plays a direct role in the recognition of substrates. In cells transformed by oncogenic Src variants, an actin-associated protein of *M_r* 110K containing proline-rich motifs (AFAP-110) is recognized by the Src SH3 domain, and phosphorylated by the kinase domain (Fig. 5b)¹⁰⁴. Tyrosine phosphorylation of AFAP-110 cements its interaction with Src by creating a binding site for the Src SH2 domain, and may target Src to actin stress fibres.

Another substrate that may interact with Src family kinases in this way is the 68K protein Sam68 (ref. 105). Sam68 binds to Src and is phosphorylated on tyrosine during mitosis, when Src itself is activated^{106,107}. Sam68 may be sequestered in the nucleus of interphase cells, and only be able to interact with c-Src on breakdown of the nuclear envelope. During mitosis,

Sam68 binds through one of its proline-rich motifs to the Src SH3 domain, subsequently becomes phosphorylated and also engages the Src SH2 domain, and may thereby mediate a mitotic-specific signal from Src^{108,109}. The nature of this signal is unknown, but as Sam68 contains a domain found in a variety of RNA-binding proteins^{108,109} and binds RNA *in vitro*, it may regulate gene expression.

The Src SH2 domain can also bind substrates without the aid of the SH3 domain. The prominent pTyr-containing protein p130^{cas}, for example, associates specifically with the SH2 domain of activated Src *in vivo*¹¹⁰. This protein is also tyrosine-phosphorylated in v-crk-transformed cells, and becomes tightly bound to the v-Crk SH2 domain, indicating that it may stimulate mitogenesis². It contains a remarkable series of motifs: an N-terminal SH3 domain is followed by 15 potential phosphorylation sites, with the consensus Tyr-X-X-Pro¹¹¹. Nine of these closely spaced motifs have the sequence Tyr-Asp-Val/Thr-Pro, which conforms reasonably well to the consensus sequence recognized by the Crk SH2 domain. Like IRS-1, p130^{cas} may therefore function as a docking protein, binding SH2-containing polypeptides through its pTyr sites. Its association with the Src SH2 domain may link it to the membrane, or may facilitate repeated phosphorylation by holding the substrate close to the kinase active site. The substrate specificities of cytoplasmic tyrosine kinases like Src, Fps and Abl generally match the binding preferences of their SH2 domains closely¹³¹, indicating that the two may well have co-evolved to allow the kinase domain to create sites recognized by its SH2 partner.

The Src SH2 and SH3 domains thus have several distinct ligands each, and can form a series of complexes with distinct functions. The choice of binding partner is regulated by Src phosphorylation, and by the location and phosphorylation state of potential binding proteins. The ability of SH2 and SH3 domains to form sequential complexes with different partners may be general, greatly expanding the scope and versatility of intracellular signalling networks. A challenge for the future will be to establish the biological significance of these complexes.

Location of signalling proteins

SH2, SH3 and PH domains all appear to control the location of signalling proteins within the cell. This is a particularly striking feature of SH3 domains, which are closely tied to the control of cell morphology. Several proteins associated with the cytoskeleton, including α -spectrin and myosin-1, possess SH3 domains. Genetic analysis in yeast has also demonstrated that SH3-containing proteins such as ABP-1, SLA1 and BEM1 are required for organization or polarization of the actin cytoskeleton^{19,112,113}. SH3-containing proteins are also important for cellular organization in *Drosophila*, where mutations in the tumour-suppressor gene *discs large* (*dlg*) lead to a loss of the tight (septate) junctions between epithelial cells and aberrant proliferation of cells in the imaginal disk¹¹⁴. The Dlg protein has a C-terminal guanylate-kinase-like domain, a central SH3 domain, and a repeated N-terminal motif. The SH3 domain is important in maintaining the cellular junction, as a substitution in this domain results in loss of function (D. Woods and P. Bryant, personal communication), and may therefore anchor junctional components to the cytoskeleton.

SH3 domains are also important for subcellular localization and cytoskeletal interactions in mammalian cells. In mammalian fibroblasts, the SH3 domains of Grb2 and PLC- γ 1 direct these proteins to membrane ruffles and actin stress fibres, respectively, indicating that their interactions control protein distribution within the cell¹¹⁵. Similarly, the SH3 domain of the cytoskeletal protein α -spectrin binds both *in vitro* and *in vivo* to a proline-rich motif in the cytoplasmic tail of an amiloride-sensitive rat sodium channel¹¹⁶. This interaction is apparently sufficient to direct the channel to the apical surface of epithelial cells.

An especially intriguing site of SH3 action is the NADPH oxidase system of phagocytic cells such as neutrophils¹¹⁷, which

is activated by inflammatory stimuli to produce superoxide, a precursor to antimicrobial oxidants. The activated oxidase complex contains a cytochrome b_{558} composed of two transmembrane proteins (gp91-phox and p22-phox), three proteins bearing SH3 domains (p47-phox and p67-phox, each with two SH3 domains, and p40-phox, with one), and a small GTP-binding protein, Rac. p47- and p67-phox are cytosolic in unstimulated cells, but move to the membrane and associate with the cytochrome b_{558} in cells stimulated by phagocytosis or inflammatory mediators. Mutations in the genes for the gp91-, p22-, p47- or p67-phox components can cause chronic granulomatous disease, a condition characterized by high susceptibility to bacterial and fungal infections.

The SH3 domains of p47-phox and p67-phox may be responsible for the interactions that assemble the functional oxidase¹¹⁸⁻¹²⁰. Recent work indicates that before stimulation, p47-phox may be in a closed conformation, with its SH3 domains engaging internal proline motifs. On activation, the C-terminal SH3 domain of p67-phox could bind specifically to the proline motif in the tail of p47-phox, while the SH3 domains of p47-phox could bind a proline-rich element in the membrane-bound p22-phox. These interactions are required for enzyme activation in intact cells. The Pro-to-Gln mutation in the SH3-binding motif of p22-phox detected in one patient with chronic granulomatous disease blocks the interaction of p22-phox with the p47-phox SH3 domains. Stimulation of phagocytic cells thus appears to activate a signalling pathway (as yet poorly defined) directed at the p47-phox and p67-phox SH3 domains, which induces a conformational change, reorganizing their SH3 ligands and recruiting the two proteins into the oxidase complex. Their function once bound to the cytochrome is unclear, although they may enhance the ability of gp91-phox to transport electrons from NADPH to O_2 . The molecular events initiating the rearrangement of the SH3 ligands in p47-phox and p67-phox are also mysterious, although the C-terminal phosphorylation of p47-phox induced by neutrophil activation may help to break the interaction of its SH3 domain with the C-terminal proline-

rich sequence. In the same way that the SH2 domains of Zap-70 mediate interactions important for T-cell activation, the SH3 domains of p47-phox and p67-phox are required for the response of phagocytic cells to pathogens.

Summary

Numerous proteins involved in signal transduction possess SH2, SH3 and PH domains, either alone or in combination. Similarly, there are many polypeptides with binding sites, such as proline motifs or tyrosine phosphorylation sites, for these modules. The resulting interactions are important for signalling from the cell surface to the nucleus, for protein trafficking and subcellular localization, for the control of cell architecture and cell-cell interactions, and for cellular responses to infection. Genetic analysis has hinted at the biological functions of these domains. Biochemical experiments now indicate that they mediate an extremely complex and versatile network of interactions which may co-ordinate cellular responses to the environment.

The use of binding modules appears to be very general in signal transduction and in control of the cell cycle. In addition to the SH2, SH3 and PH domains, recently discovered modules include the PTB domain; the LIM domain, a cysteine-rich, zinc-binding unit which appears to regulate protein-protein interaction in a variety of transcription factors, cytoskeletal proteins and other signalling polypeptides¹²¹; the armadillo (arm) domain, a common element of proteins such as β -catenin which form part of cellular junctions and are involved in both cell adhesion and in cell-cell signalling¹²²; and the Notch/ankyrin repeat, which is present in transmembrane receptors, cytoskeletal proteins and transcription factors, and mediates protein:protein interactions crucial for intercellular signalling¹²³. Remarkably, a cytoplasmic tyrosine kinase similar to Zap-70 has been described in the simple metazoan *Hydra vulgaris*, in which the two SH2 domains flank five ankyrin repeats¹²⁴. The plot thickens. □

Tony Pawson is in the Division of Molecular and Developmental Biology, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario M5G 1X5, Canada.

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ARTICLES

Crystal structure of the nickel–iron hydrogenase from *Desulfovibrio gigas*

Anne Volbeda, Marie-Hélène Charon, Claudine Piras,
E. Claude Hatchikian*, Michel Frey† & Juan C. Fontecilla-Camps†

Laboratoire de Cristallographie et de Cristallogénèse des Protéines, Institut de Biologie Structurale J. P. Ebel (CEA,CNRS),
41 avenue des Martyrs, 38027 Grenoble Cedex 1, France

* Unité de Bioénergétique et Ingénierie des Protéines, CNRS, 13402 Marseille Cedex 20, France

The X-ray structure of the heterodimeric Ni–Fe hydrogenase from *Desulfovibrio gigas*, the enzyme responsible for the metabolism of molecular hydrogen, has been solved at 2.85 Å resolution. The active site, which appears to contain, besides nickel, a second metal ion, is buried in the 60K subunit. The 28K subunit, which coordinates one [3Fe–4S] and two [4Fe–4S] clusters, contains an amino-terminal domain with similarities to the redox protein flavodoxin. The structure suggests plausible electron and proton transfer pathways.

THE capacity to oxidize molecular hydrogen or reduce protons ($\text{H}_2 \rightleftharpoons 2\text{H}^+ + 2\text{e}^-$) is a central metabolic feature of a wide variety of microorganisms¹. H_2 oxidation is coupled to the reduction of electron acceptors such as oxygen, nitrate, sulphate, carbon dioxide and fumarate, whereas proton reduction (H_2 evolution) is an essential element of pyruvate fermentation or the disposal of excess electrons. A H_2 -cycling process that would contribute to the generation of a proton gradient during growth on organic substrates in the presence of sulphate has been reported in some sulphate-reducing bacteria². Three types of metal-containing hydrogenases, the enzymes responsible for hydrogen metabolism in microorganisms, have been defined: 'iron only' or Fe hydrogenases³, Ni–Fe hydrogenases and Ni–Fe–Se hydrogenases^{4–6}.

The Ni–Fe hydrogenase from *Desulfovibrio gigas* is a heterodimeric periplasmic protein consisting of 28K and 60K subunits^{7,8}. The latter subunit matures to a functional form through cleavage of its 15 C-terminal residues⁹. Besides the nickel ion¹⁰, *D. gigas* hydrogenase contains three iron–sulphur centres⁷. These centres have been identified as one [3Fe–4S] and two [4Fe–4S] clusters¹¹. The enzyme isolated in aerobic conditions exhibits two Ni-related electron paramagnetic resonance (EPR) signals, called Ni-A and Ni-B^{12,13}. The Ni-A signal corresponds to the so-called 'unready' state of hydrogenase whose complete activation may take several hours. The Ni-B signal corresponds to the 'ready' state, which can be quickly reduced to the active form by H_2 under anaerobic conditions thereby developing H_2 -uptake activity in the presence of electron acceptors^{12,13}. During reduction in the presence of hydrogen, the enzyme displays a light-sensitive EPR signal called Ni-C, which

† To whom correspondence should be addressed.

TABLE 1 X-ray structure determination

Data and MIR phasing statistics	Native	KAu(CN) ₂	EMTS*	K ₂ PtCl ₄ (a)	K ₂ PtCl ₄ (b)	K ₂ PtCl ₄ (c)	PrCl ₃
Data set	Native	KAu(CN) ₂	EMTS*	K ₂ PtCl ₄ (a)	K ₂ PtCl ₄ (b)	K ₂ PtCl ₄ (c)	PrCl ₃
Crystals	6	1	1	1	1	1	1
Measured <i>h</i> <i>k</i> <i>l</i> s	11,6003	43,538	25,465	17,913	7,119	23,407	24,093
Unique <i>h</i> <i>k</i> <i>l</i> s	31,053	23,390	17,783	10,178	6,331	17,251	17,983
Resolution (Å)†	2.85	3.2 (4.4)	3.3 (4.8)	4.0 (4.3)	4.0 (4.3)	3.2 (4.5)	3.3 (—)
Completeness (%)	85.5	91.2	76.1	77.7	48.4	67.2	77.0
<i>R</i> _{merge} (%)‡	8.6	7.0	7.8	8.5	7.4	9.0	8.2
<i>R</i> _{der} (%)§		12.7	18.7	26.7	22.1	22.3	14.8
Binding sites		2	4	10	8	8	1
Phasing power		0.75	0.81	1.38	1.39	0.95	0.28
<i>R</i> _{cutis} (%)¶		0.86	0.86	0.69	0.71	0.82	0.98
Refinement statistics (including r.m.s. deviations with respect to 'ideal' or dictionary values)							
κ (°) and T (Å) of non-crystallographic symmetry operation#						124.4 14.0	
Total number of non-hydrogen atoms (2 independent hydrogenase molecules)						12,288	
Included water molecules						28	
Average temperature factor (in Å ² , for molecules 1 and 2)						17.6 20.4	
<i>R</i> -factor** (in %, using 29,660 <i>F</i> _{obs} , 2.85–8.0 Å resolution range, no σ-cutoff)						17.8	
Bond length distances (Å)						0.012	
Bond angles (°)						3.4	
Planarity of planar groups (Å)						0.011	
B-factor correlations: main chain bond, angle; side chain bond, angle (Å ²)						0.7 1.2 1.1 1.9	

The conditions to grow the triclinic crystals of *D. gigas* hydrogenase used in this study were first obtained by C. Abergel and later optimized by us (manuscript in preparation). Their cell dimensions are *a* = 63.6 Å, *b* = 93.9 Å, *c* = 69.2 Å, *α* = 89.8°, *β* = 102.9°, *γ* = 90.8°, and they contain two molecules per unit cell and ~41% solvent. The structure was solved using the multiple isomorphous replacement (MIR) method, combined with density averaging. All data sets but one were collected with a Siemens/Xentronics area detector using a rotating anode source, and processed with the program XDS³⁵. One partial native data set was collected with an image plate detector using synchrotron X-rays produced at LURE, Paris, and processed with MOSFLM³⁶. Further data reduction and scaling was done with the BIOMOL and CCP4³⁷ crystallographic packages. Heavy-atom sites were located by Patterson and difference Fourier methods. Their positions and occupancies were refined with the program MLPHARE^{38,39}. A PrCl₃ derivative showing a single binding site was used only for the location of the most occupied binding sites of the other three derivatives¹⁹. As the MIR phase information was rather poor beyond 5 Å resolution, phases were extended from 5 to 3.3 Å in 30 small steps each consisting of 10 cycles of 2-fold density averaging using the DEMON package⁴⁰; the two protein envelopes were obtained by density correlation techniques. MIR phases were combined only in the new resolution shell added in each extension step. At the end, phases were extended in 3 steps from 3.3 to 3.21 Å resolution without phase combination. Chain tracing and model building was done with programs FRODO⁴¹, O⁴² and TURBO⁴³ on ESV and IRIS workstations. The model was refined with XPLOR⁴⁴ and PROLSQ⁴⁵, using non-crystallographic symmetry restraints in the initial stages at 3.2 and 2.85 Å resolution, but not in the final stages. Individual isotropic temperature factors were also refined. Finally, an almost complete model was obtained, missing only six N-terminal residues of the large subunit and three N-terminal residues of the small subunit.

* Ethyl mercury thiosalicylate.

† The number in brackets is the highest resolution for which the phasing power is higher than 1.0. Note that the native data are 48% complete between 3.0 and 2.85 Å resolution.

‡ $R_{\text{merge}} = \sum_{hkl} \sum_i |I_{hkl,i} - \langle I_{hkl} \rangle| / \sum_{hkl,i} \langle I_{hkl} \rangle$, using Friedel mates as independent observations.

§ $R_{\text{der}} = \sum_{hkl} |F_{\text{der}} - F_{\text{nat}}| / \sum_{hkl} F_{\text{nat}}$.

|| Phasing power is defined as $\langle F_H / \text{lack of closure} \rangle$.

¶ $R_{\text{cutis}} = \langle \text{lack of closure} \rangle / \langle \text{isomorphous difference} \rangle$, as defined in MLPHARE for acentric reflections.

κ, Rotation angle (°); T, translation (Å) parallel to rotation axis.

** The crystallographic *R*-factor is defined as $\sum_{hkl} |F_{\text{obs}} - F_{\text{calc}}| / \sum_{hkl} F_{\text{obs}}$.

decays upon photolysis at a temperature of 77 K, generating another EPR signal, Ni-L or Ni-C*. Because the rate of photolysis is much slower in D₂O than in H₂O, it has been postulated that Ni-C represents a nickel-hydrogen species¹⁴, possibly a Ni(II)-hydride^{15,16}. Two EPR-silent redox states, each arising from the one-electron reduction of Ni-B and Ni-C, have also been reported^{12,13,17}. These states have been called Ni-SI and Ni-R¹⁸. In addition to the Ni ion, Fe-S clusters have been characterized at various stages of the reductive activation process by EPR and Mössbauer studies^{11,17}. The midpoint redox potentials for the two [4Fe-4S] clusters are closely related to the corresponding values for the different nickel states¹⁷, suggesting that these clusters function as ancillary redox centres. By contrast, the involvement of the [3Fe-4S] cluster in electron transfer has been questioned, both because of its relatively high midpoint redox potential, and the fact that it seems to be absent from the Ni-Fe-Se hydrogenases, which are otherwise very similar to the Ni-Fe enzymes. The Fe-S clusters and the Ni binding site were initially located by us during the preliminary X-ray analysis at 5 Å resolution¹⁹. More recently, a very similar distribution of the four metal sites has been observed at 4 Å resolution in *Desulfovibrio vulgaris* (Miyazaki) hydrogenase²⁰.

We report here the three-dimensional structure of the Ni-Fe hydrogenase from *D. gigas* as determined by X-ray crystallography to a resolution of 2.85 Å. This study gives a detailed account of the positions and coordinations of the three iron-sulphur centres of the enzyme, provides the first description of the organization of a nickel-containing active site in an enzyme and shows that, for the most part, *D. gigas* hydrogenase is the prototype

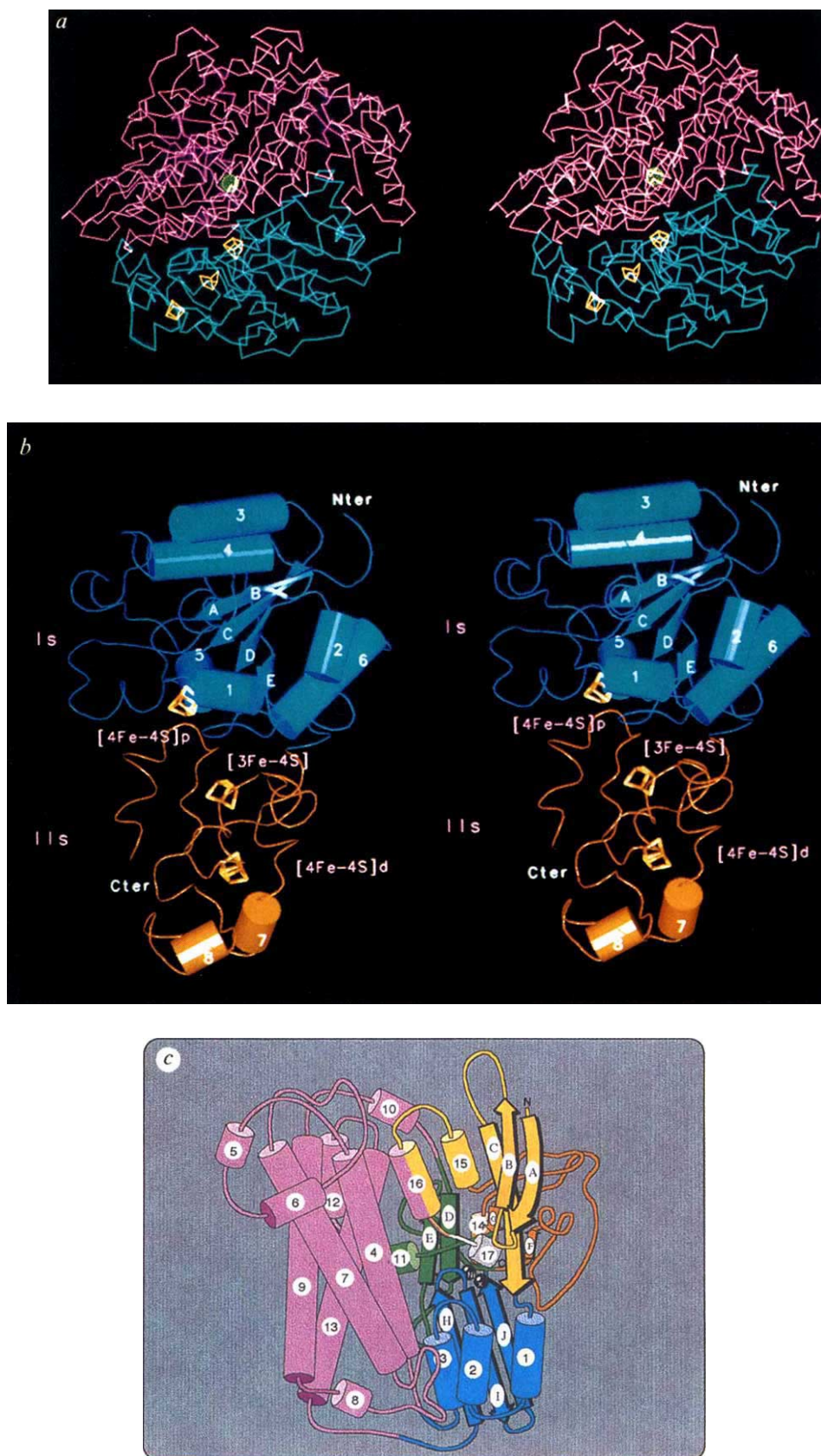
of a new topological class of protein molecules. The hydrogenase model presented here provides the first structural basis for understanding at the atomic level how molecular hydrogen is used or produced by microorganisms.

Structure description

The structure of *D. gigas* hydrogenase was solved as described in Table 1. The model refined at 2.85 Å resolution has close-to-ideal geometry and shows a good fit to the electron density map. The two hydrogenase subunits (Fig. 1*a-c*) interact very extensively, with a contact surface of about 3,500 Å², forming a globular heterodimer. The amino-acid sequence of the two subunits and corresponding secondary structure elements are given in Fig. 1*d, e*. The small subunit binds three Fe-S clusters. Searches of the Brookhaven protein data bank²¹ indicate that the large subunit, which contains the Ni binding site, is topologically unique.

In the small subunit, the *α/β* twisted open-sheet structure of the N-terminal I₅ domain (Fig. 1*b*) is very similar to that of the redox protein flavodoxin. Eighty-nine out of the 136 residues of *Clostridium MP* flavodoxin, including 5 *β*-strands and five helical regions²², can be superimposed onto the I₅ domain with a r.m.s. deviation of 2.7 Å for equivalent C α -atoms. Also, the binding sites for the phosphate moiety of the flavin mononucleotide and the [4Fe-4S] cluster closest to the Ni ion (see below) can be superimposed and are topologically equivalent. Manual optimization of the amino-acid sequence alignment obtained from the three-dimensional superposition (not shown) gives between 15 and 20% of amino-acid sequence identity and hints at a possible evolutionary relationship between the two proteins.

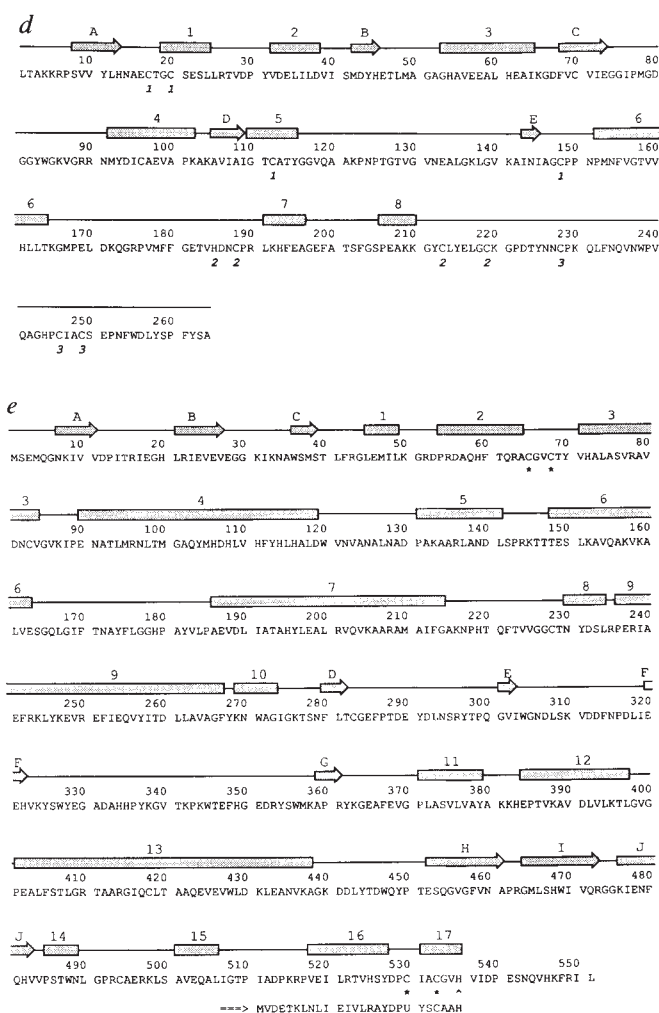
FIG. 1 Structure and amino-acid sequence⁸ of *Desulfovibrio gigas* hydrogenase. Secondary structure elements were located with DSSP⁴⁶. Helices are shown as cylinders and numbered. β -strands are depicted by arrows and labelled with capital letters. *a*, α -tracing. The small and the large subunit are depicted in blue and pink, respectively. The four metal centres are highlighted: the Ni-containing active site as a dotted green sphere inside the large subunit, the three Fe-S centres in yellow in a sequential arrangement in the small subunit (this figure and Fig. 4a were made with FRODO⁴¹). *b*, The small subunit, shown with its contact interface with the large subunit facing the reader. The N-terminal domain 1_s (depicted in blue) binds the closest Fe-S cluster relative to the active site, here labelled [4Fe-4S]_p. The C-terminal domain 2_s (shown in red) contains the [3Fe-4S] cluster and the most distal cluster from the active site, here labelled [4Fe-4S]_d (this figure, as well as Figs 2 and 3a, were made with the program O⁴²). *c*, The large subunit. Five structural domains can be distinguished made of non-sequential polypeptide regions brought together by the overall protein folding. These include three $\alpha\beta$ -type domains (shown in yellow, blue and green), a large α -helical domain (shown in pink) containing a very long four-helical bundle, and a fifth less structured domain (shown in orange). Following the polypeptide chain, the large subunit appears to be organized in three layers running roughly parallel to the subunit interface. Two metal ions, Ni and X (proposed to be Fe) are found in the buried active site. *d*, Amino-acid sequence of the small subunit. Metal ligands of [4Fe-4S]_{prox}, [4Fe-4S]_{dist} and [3Fe-4S] are labelled 1, 2 and 3, respectively. *e*, Amino-acid sequence of the large subunit. Active-site metal ligands are indicated by *, the crystallographically observed C-terminal residue by ^, and the aligned amino-acid sequence of the very small subunit of the F420 non-reducing hydrogenase of *Methanococcus voltae*²⁶ (U represents a selenocysteine) by $\Rightarrow$.



Iron-sulphur clusters

The three Fe-S clusters contained in the small subunit of *D. gigas* hydrogenase are distributed almost along a straight line, with the [3Fe-4S] cluster located half-way between the two [4Fe-4S] clusters. The two [4Fe-4S] clusters have been named

proximal (prox) and distal (dist) based on their distance to the Ni ion. The first four cluster ligands (the conserved cysteines 17, 20, 112 and 148) are found in domain I_s (Fig. 1b) and bind [4Fe-4S]_{prox}; the next four (His 185 and cysteines 188, 213 and 219) are part of domain II_s and bind [4Fe-4S]_{dist} (Fig. 2a),



whereas the last three (cysteines 228, 246 and 249, also from domain II_S) bind the [3Fe-4S] cluster (Fig. 2b). This is the first time that a histidine side chain has been observed as a [4Fe-4S] cluster ligand in a protein structure. Its imidazole ring is located on the surface of the molecule, where it could participate in electron transfer to and from the reported²³ redox partner of hydrogenase, cytochrome *c*₃.

According to analyses of amino-acid sequence homologies^{24,25} the N-terminal domain I_S is found in the small subunit of most of Ni-containing hydrogenases, suggesting that the proximal [4Fe-4S] cluster is an almost invariant feature. Several residues from the large subunit interact with the [3Fe-4S] cluster (Lys 216 and Glu 221) and with [4Fe-4S]_{prox} (Arg 63 and His 219). The latter two residues are the most conserved ones, also pointing at [4Fe-4S]_{prox} as being the most conserved cluster.

Hydrogenase maturation

The chain tracing of the large subunit in the electron density map could not be extended beyond His 536 (Fig. 3a), which is 15 amino-acid residues from the C-terminal Leu 551 (Fig. 1e), as determined by gene sequencing⁸. This agrees with reports concerning the functional maturation of the large subunit which is mediated by the cleavage of these last 15 residues⁹. Subsequently, a mass spectrometric analysis of the mature subunit gave a relative molecular mass (*M*_r) of 59,459 ± 6, which is close to the theoretical value of 59,704 calculated after omitting the cleaved peptide (Fig. 1e). Considering the precision inherent to mass spectrometry, the difference of 245 between the given values may represent minor errors or deletions in the amino-acid sequence.

Similar C-terminal processing has been found to take place in other hydrogenases, including the 25-residue-long third subunit

of the F420 non-reducing Ni-Fe-Se enzyme from *Methanococcus voltae*²⁶. In the crystal structure of *D. gigas* hydrogenase the region corresponding to this small third subunit is formed by the buried C-terminal α -helices 16_L and 17_L (Fig. 1c, e). Many conserved residues interact with these two helices (Fig. 3b). Because α -helix 17_L appears to be directly involved in the binding of Ni (see below), a plausible general maturation mechanism of Ni-Fe and Ni-Fe-Se hydrogenases can be described as consisting of proteolytic cleavage after a specific site (His 536 in *D. gigas*), and structural reorganization of the molecule including the formation of the metal-containing active site.

The Ni-binding site

This study shows that the active site contains two metal ions (at the sites labelled M1 and M2 in Fig. 4a). Whereas the thiolates from cysteines 68 and 533 serve as bridging ligands of the two metal ions, Cys 65 and Cys 530 are bound to the metal at M2 exclusively. M1, the other metal position in the active site, displays a strong peak in the anomalous difference map (Fig. 4a). It is highly unlikely that this signal originates from Ni because this metal is a very weak anomalous scatterer of Cu-K α radiation. This provides indirect evidence that Ni binds at site M2. Additional support for the assignment of Ni to M2 comes from the fact that spectroscopic studies have shown that in Ni-Fe-Se hydrogenases the selenocysteine homologous to Cys 530 found at site M2 is a Ni ligand²⁷⁻²⁹.

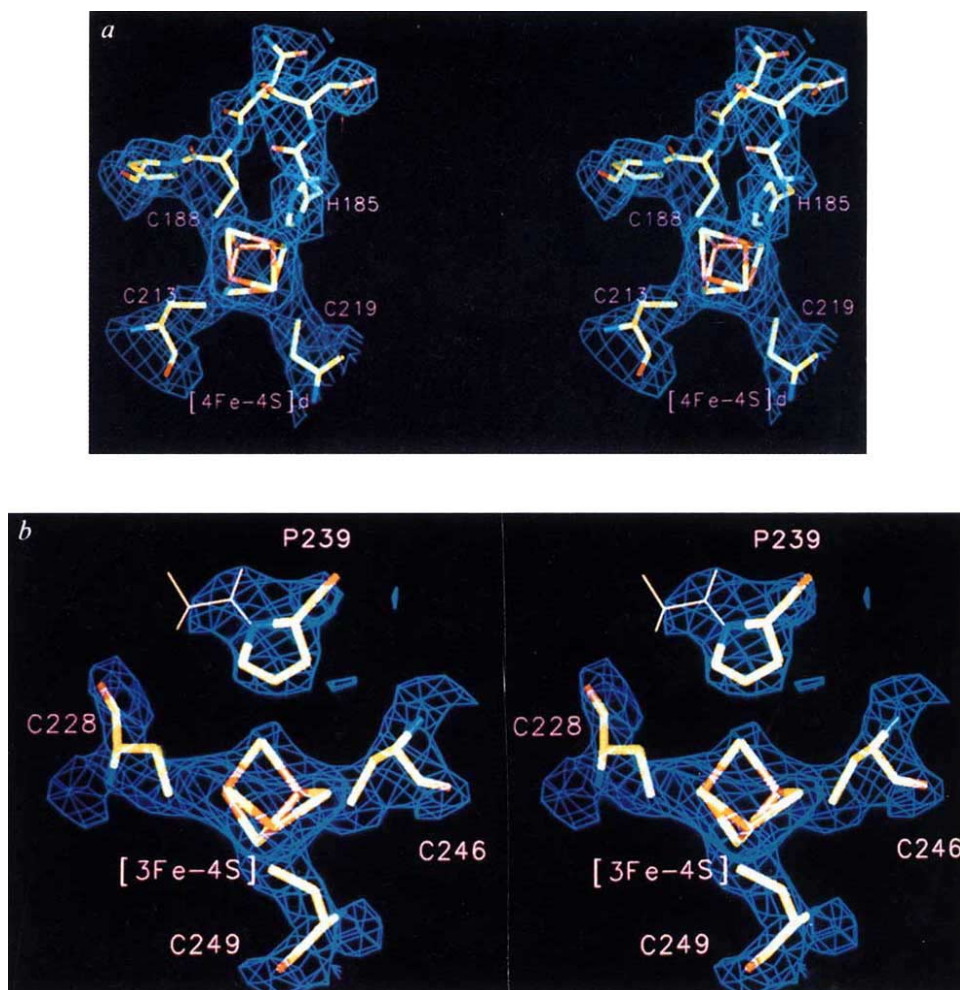
The nature of the metal (labelled X in Fig. 4b) at site M1 remains uncertain. In addition to Ni, Cu, Zn, Ca and Mg can also be excluded because they are weak anomalous scatterers of Cu-K α radiation. Potential candidates include Fe, Co, Mn and V which are strong anomalous scatterers and have all been found to occur in proteins. Mo could also be included in this list, but the fact that it has many more electrons than the other metals and therefore should give a significantly higher peak argues against it. A recent inductively coupled plasma (ICP) analysis of samples similar to the ones used in the crystallization experiments has shown that the protein contains neither Mn nor V. A small amount of Co was found, equivalent to less than 20% of the 1.04 ± 0.1 atoms of Ni determined per molecule. However, this quantity is not sufficient to explain the high peak at site M1. Consequently, and because hydrogenase of *D. gigas* has been previously reported to contain 12 ± 1 iron atoms per molecule⁷ (a result confirmed by the present ICP analysis), the most likely candidate for metal X is Fe. On the basis of these observations, the metal at site M1 has been tentatively included in the current atomic model as a fully occupied Fe ion.

In the crystal structure the temperature factors of the Ni and its S_γ ligands refined to rather high values compared to those of neighbouring atoms (Fig. 4b). In principle this could be due to disorder, X-ray damage, partial Ni occupancy or the presence of several Ni redox states. EPR analysis of hydrogenase crystals obtained under the conditions used in the crystallographic analysis showed the Ni signal to be a mixture of about 85% Ni-A and 15% Ni-B. It should be noted that the EPR-detectable Ni-A signal has been reported to correspond to only about half of the estimated metal content of 0.95 Ni atoms per hydrogenase molecule as determined by atomic absorption¹⁰. The nature of the EPR silent fraction is not known. It is clear that it does not correspond to a 'ready' or active form of the enzyme because the development of full hydrogen uptake activity (502 μ mol H₂ min⁻¹ mg⁻¹) of these samples takes hours. Taken together, these observations suggest that the observed electron density corresponds to a disorganized state of the active site, a significant fraction of which is responsible for the Ni-A signal.

Electron and proton transfer

The observed spatial arrangement of the three Fe-S clusters in *D. gigas* hydrogenase provides one plausible electron channel going from the active site to the molecular surface (Fig. 5a).

FIG. 2 Structure of two Fe-S clusters fitted to the electron density calculated with $2mF_o - DF_c$ map coefficients⁴⁷. **a**, The $[4Fe-4S]_{dist}$ cluster, including the exposed His 185 that functions as a ligand to one of the four Fe ions. In some Ni-containing hydrogenases this histidine is substituted by a cysteine^{24,25}. **b**, The $[3Fe-4S]$ cluster, highlighting the cysteine ligands of the three Fe ions, and the proximity of Pro 239 with respect to the empty potential fourth Fe site.



Furthermore, a crown of acidic residues surrounds the partially exposed His ligand of $[4Fe-4S]_{dist}$ and this might provide a recognition site for the reported²³ redox partner cytochrome c_3 . Still, the role of the $[3Fe-4S]$ cluster is not clear. Although its position suggests an active role in electron transfer, the high redox potential of this cluster and its absence from some hydrogenases cast doubts about its involvement in electron transport in *D. gigas* hydrogenase. Note that Pro 239, which points at the empty potential fourth Fe-site of the $[3Fe-4S]$ cluster (Fig. 2b), is substituted by a cysteine in the Ni-Fe-Se hydrogenase from *D. baculatus*⁸. This and the absence of its typical EPR signal suggests that the $[3Fe-4S]$ cluster has become a $[4Fe-4S]$ cluster in the *D. baculatus* enzyme. Thus, it appears that alternative electron pathways may exist in different classes of Ni-containing hydrogenases.

Another important functional aspect of hydrogenase is how the enzyme disposes of protons formed during hydrogen oxidation, or how they get to the active site for the reverse reaction. Four histidines and a partially exposed glutamate have been located between the active site and the molecular surface (Fig. 5b). Histidines and glutamates have suitable pK values for transferring protons. Moreover, the glutamate and three of the four histidines are highly conserved among Ni-Fe and Ni-Fe-Se hydrogenases^{24,25}. Therefore, a pathway may be proposed for the transfer of protons, first from the active site to His 72 and next from His 72 to His 536 through a flip-flop mechanism of the imidazole ring, and finally from His 536 through ordered water molecules and Glu 46 to the solvent medium. His 468 and His 482 might be involved as well. It should be noted, however, that the active site is inaccessible to solvent protons when the enzyme is in the oxidized Ni-A state^{15,30}. The reasons for this

are not yet understood and are not immediately obvious from the crystal structure.

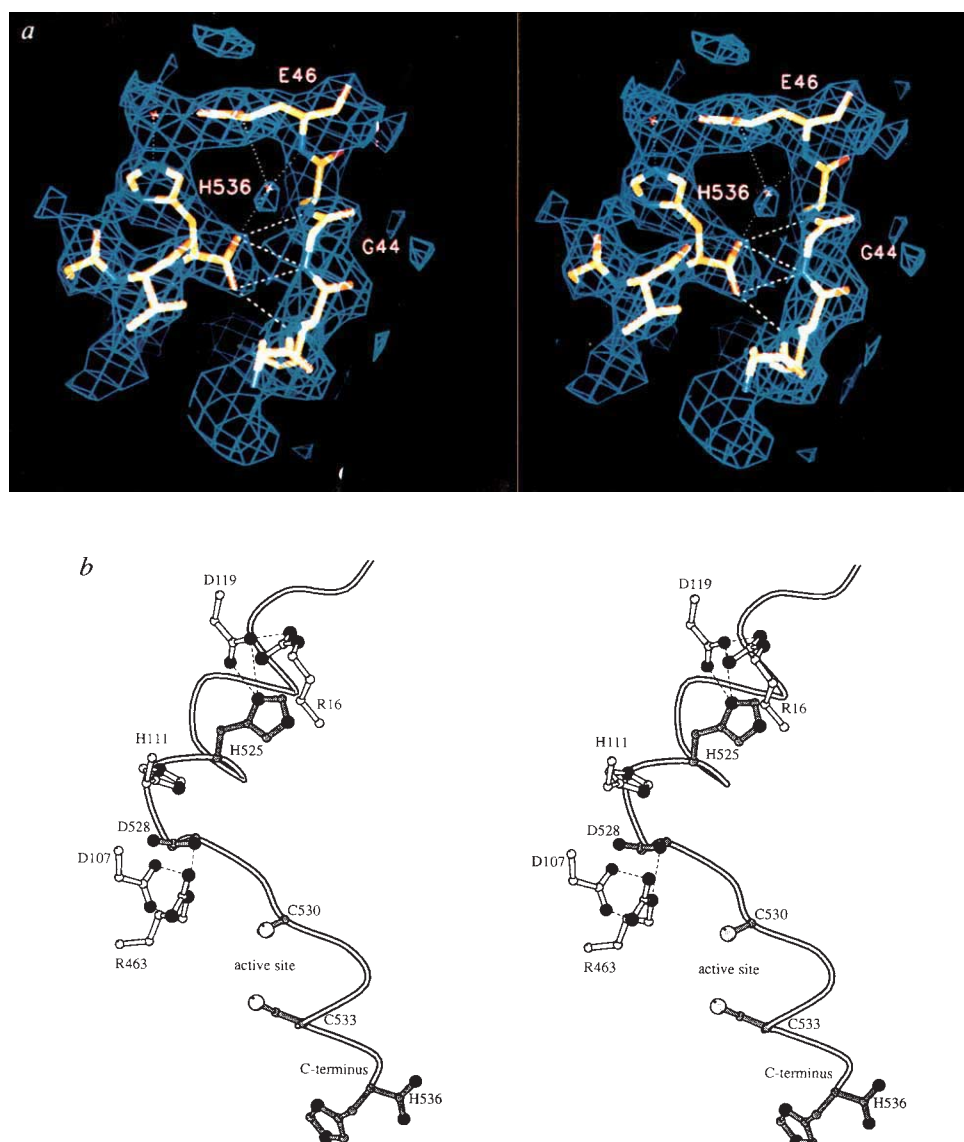
Possible pathways for H_2 have not been identified in the crystal structure. It is likely that, as in the case of CO access to the haem group of myoglobin³¹, several dynamic pathways exist for molecular hydrogen that depend on fluctuations in the protein conformation.

The active site of hydrogenase

The crystallographic analysis of hydrogenase has shown that its active site is buried in the large subunit and contains two metal ions, one of which is Ni. The identity of the second metal ion, here called X, remains uncertain although, as argued above, Fe appears as the most likely candidate. Additional support for the presence of an Fe ion at the active site comes from very recent EXAFS studies (S. Wang, R. A. Scott, R. Franco, I. Moura, J. J. G. Moura, J. Le Gall & A. E. Przybyla, personal communication) which have suggested the existence of an Fe ion at 2.55 Å from the Ni atom in the activated forms of the enzyme.

The presence of a possible Ni-Fe centre would require a re-interpretation of the EPR Ni signals that have usually been assigned to a mononuclear site. Although such reinterpretation may have to invoke some unusual chemistry, one must bear in mind that the geometry of the active site may be greatly conditioned by the protein environment. As an example, only four thiolates may serve as Ni protein ligands in the oxidized hydrogenase, resulting in an uncommon coordination geometry that is neither square planar nor tetrahedral (Fig. 4b). It should also be mentioned here that although the three non-protein ligands of X have been modelled as water, their nature is not known at this time. This is relevant because ligands can influence the spin

FIG. 3 The C-terminal region of the large subunit. **a**, Fit of the buried C-terminal His 536 to the $2mF_o-DF_c$ electron-density map, showing two putative water molecules and, in dashed lines, a number of possible hydrogen bonds. **b**, Possible conserved packing interactions of the C-terminal region (shaded amino acids), with the rest of the large subunit. For clarity, the interactions of the two active site cysteine residues contained in helix 17_L are omitted (see Figs 4 and 5). The two C-terminal helices serve as central elements in the folding of the large subunit, 16_L packing against the long four-helical bundle and the N-terminal three-stranded A_L-C_L β -sheet, and 17_L stabilized by a pocket formed by residues from the yellow, blue and orange domains shown in Fig. 1c. These two helices are part of an independent 25-residue-long third subunit in the F420 non-reducing hydrogenase from *M. voltae*²⁶ (Fig. 1e). This figure and Figs 4b and 5 were made with the program MOLSCRIPT⁴⁸.



state of metal ions and, consequently, their spectroscopic properties. In order to involve other residues in the coordination of the metal ions in the activated forms of the enzyme, major movements of either the metal ions and/or main chain atoms would be required upon activation.

The existence of a two-metal cluster at the active site raises the possibility that, upon reductive activation, a three-centre metal-hydrogen-metal (MHM) bond could be formed. MHM bonds are quite common in compounds containing two or more transition metal ions³², and the stereochemistry of the heterodinuclear site in the crystal structure of hydrogenase is compatible with such a bond: upon binding of the H^- , the coordination sphere of X would become octahedral or tetragonal, and that of the Ni ion, square pyramidal. (Fig. 4b). Such a hydride-metal complex could correspond to an intermediate state of the reaction or, alternatively, to the actual active site. CO, a competitive inhibitor of hydrogenase, might also act as a bridging ligand of the two active site metal ions as it has been observed for model compounds³². Thus, the reversible photolytic effect on the CO-Ni bond³³ might result in the formation of a transient terminal X-CO bond. The same process would apply to Ni-L¹⁴ or Ni-C* (ref. 13), giving a terminal X-hydride bond instead. Given their proximity to the putatively active Ni ion, one or several of the thiol groups may be 'non-innocent' ligands and play a role during catalysis as well. For instance, in Ni-Fe-Se hydrogenases,

the natural substitution of Cys 530 by a selenocysteine affects H_2 :HD exchange ratios⁴.

Intriguingly, X-ray absorption spectroscopy reports on *Thiobacillus roseopersicina* hydrogenase suggest that the overall electron density at the Ni site does not change during redox titration³⁴ implying that this ion is not directly responsible for H_2 oxidation. To what extent a heterodinuclear site may account for these observations is not clear at this time but the possible involvement of a second redox active metal ion seems worth exploring.

The presence of hydrogenases in many strictly anaerobic bacteria can be explained by the central role played by molecular hydrogen as an intermediate in fermentation processes. On the basis of our results, the most parsimonious mechanism of action for the hydrogenase from *D. gigas* involves transit of electrons, generated by the oxidation of H_2 , from the two-metal active site to the distal [4Fe-4S] cluster through the other two Fe-S clusters, and then to cytochrome c_3 . Protons generated as by-products of the reaction, would be evacuated through a specific transfer pathway containing a highly conserved His/Glu motif. Obviously, a clearer picture of the mode of action of hydrogenase will emerge when the crystallographic study is extended to well defined homogenous redox states of the enzyme. Of special interest is the structural analysis of Ni-CO complexes because they should provide an indirect image of the otherwise elusive

FIG. 4 The active site of hydrogenase. **a**, Experimental evidence for the presence of two metal ions. A 2.85 Å resolution $F_o - F_c$ omit map is shown in purple, contoured at the 4 sigma level. Phase information comes from a model that contains neither the S_γ atoms of cysteines 65, 68, 530 and 533 nor the metal ions. The highest feature of this map (18.0 σ) coincides with a significant peak (5.7 σ) in a 4.5 Å resolution anomalous difference map (shown in red), suggesting the presence of a metal X, tentatively assigned as Fe, at the site called M1. After inclusion of an Fe at this site in the model, a next omit map (shown in blue, contoured at the 6 σ level) showed a second metal, assigned to Ni, at site M2, about 2.7 Å away from M1. Subsequent omit maps showed the positions of the S_γ atoms of the four invariant cysteines and of three additional non-protein ligands. **b**, Observed coordination of the two metal centres. The S_γ of Cys 530 is in an apical position at ~ 2.6 Å from the Ni, which may be compared to an average distance of ~ 2.25 Å for the three equatorial Cys ligands. Metal X is bound by three putative water molecules at ~ 2.05 Å, and by only two protein ligands which bridge the two metals, Cys 68 S_γ at ~ 2.35 Å and Cys 530 S_γ at ~ 2.15 Å. The Ni coordination site labelled I is not occupied by any protein ligand. In the current model this site, which is also in a coordination position for metal X, is empty. Significant disorder is indicated by the temperature factors of the active site atoms in the two independent hydrogenase molecules, ranging from 12.0 to 27.0 (in Å²) for the S ligands, from 5.2 to 17.3 for the modelled water ligands, and from 12.0 for metal X (modelled as Fe) to 27.6 for the Ni.

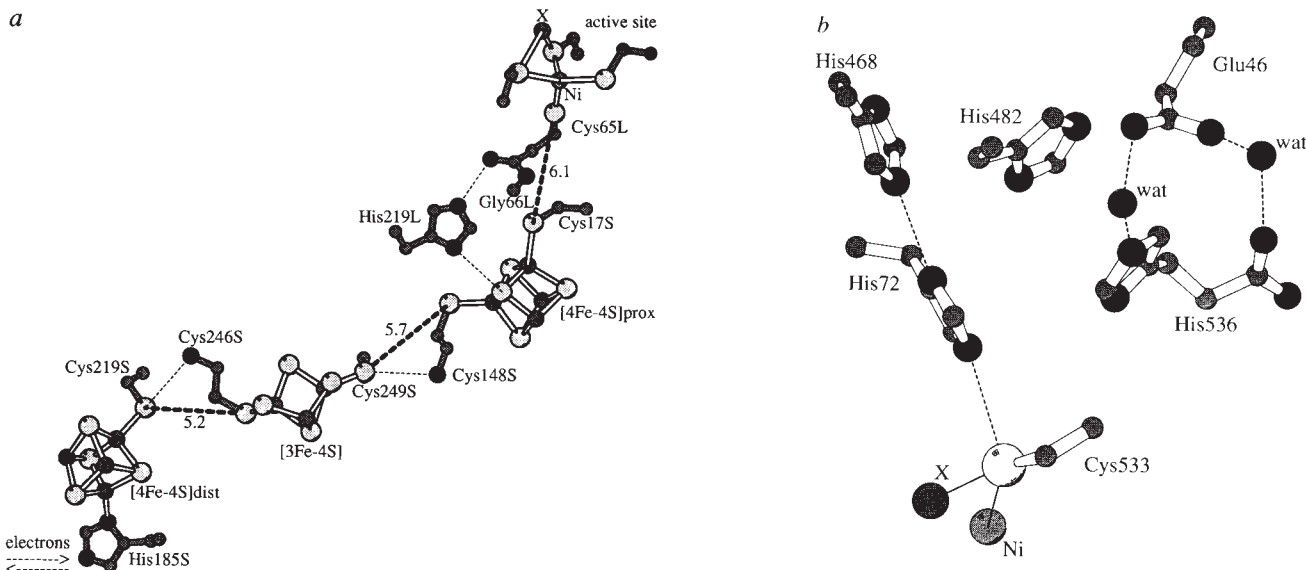
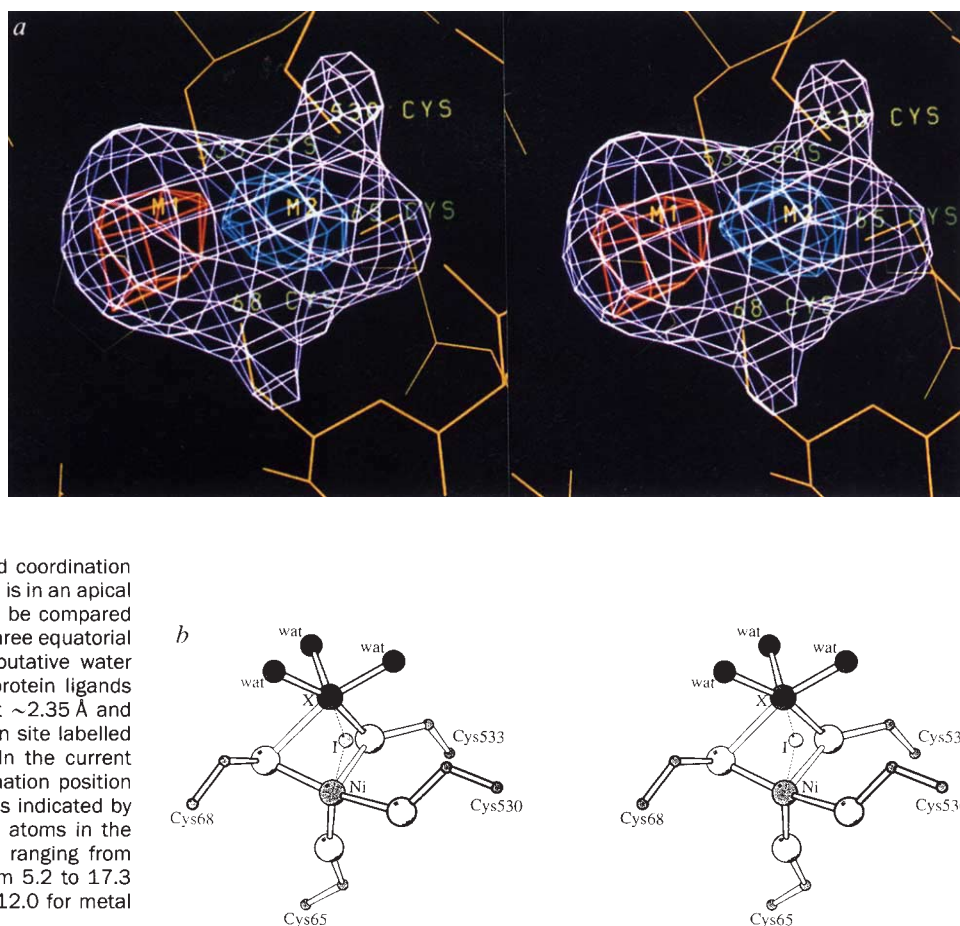


FIG. 5 Electron and proton transfer in the large (L) and small (S) subunit. **a**, Residues and metal centres involved in a possible electron transfer pathway from the buried active site to the exposed His 185_S. The shortest path⁴⁹ would include, besides the indicated H bonds (thin dashed lines) and metal-ligand interactions, covalent bonds of Cys 65_L, His 219_L, Cys 148_S, Cys 246_S and His 185_S. An alternative pathway between the Ni and [4Fe-4S]_{prox} would involve a 'through space' jump from Gly 66_L to the S_γ -atom of Cys 17_S. The numbers next to the thick dashed lines give the nearest edge-to-edge distance (in Å) between subsequent redox centres, assuming that the S_γ atoms of cysteine ligands may be counted as part of the respective redox centres¹⁶. The nearest S_γ -to- S_γ distance between the two [4Fe-4S] clusters in the

crystal structure is 16.4 Å. Using the organic glass model of protein-mediated electron transfer⁵⁰, this would give a maximal electron transfer rate between these two clusters in the order of 10^5 s⁻¹, almost two orders of magnitude higher than the maximal hydrogen turnover rate of the enzyme⁵. Thus, the [3Fe-4S] cluster may not be absolutely required for efficient electron transfer from the active site to the distal cluster, although the detailed implications of its position in between the two [4Fe-4S] clusters are unclear. **b**, Four buried histidines, one partially exposed glutamic acid and two water molecules possibly involved in a proton transfer pathway from the active site to the surface of *Desulfovibrio gigas* hydrogenase. Except for His 482 all the residues depicted are highly conserved^{24,25}.

metal-hydride complexes. Also, gas accessibility to the active site could be probed by collecting X-ray data on crystals exposed to a high-pressure Xe atmosphere because Xe can be detected in difference Fourier electron density maps. These studies are underway. □

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LETTERS TO NATURE

Discovery of explosion fragments outside the Vela supernova remnant shock-wave boundary

B. Aschenbach, R. Egger & J. Trümper

Max-Planck-Institut für extraterrestrische Physik, D-85740 Garching, Germany

THE nearby (500 pc) Vela supernova remnant has presented several puzzles. Its true shape has been difficult to determine¹, and as a result it has never been clear whether the pulsar seen in that part of the sky resulted from the same explosion that created the remnant². Here we present an X-ray image of the Vela supernova remnant which shows that the pulsar is close to the centre, and therefore most probably resulted from the same explosion. We see six extended X-ray features outside the blast-wave front, which apparently originated close to the pulsar. We propose that these features result from the passage through the surrounding medium of fragments formed by instabilities during the collapse and subsequent explosion of the progenitor star.

The constellation Vela was scanned by the Rosat X-ray telescope during the all-sky survey between October 1990 and January 1991. The image including the Vela supernova remnant (SNR) region is shown in Fig. 1. Before the Rosat observations, only the X-ray-brightest parts, marked in red and white in Fig. 1,

were known. The Rosat image now reveals that the SNR is much larger at lower surface brightness levels. The faint western parts are crossed by a long arc-shaped filament, previously noticed by Seward³ who associated the arc with the Vela SNR based on its Vela-type X-ray spectrum. Figure 1 shows that Vela extends significantly even beyond the filament towards the west. At an even lower surface brightness level of 7×10^{-15} erg cm⁻² s⁻¹ arcmin⁻² (~500 times less than the brightest patch in the north of the remnant) a faint shell appears, the contour of which is well matched by a circle of 8.3° or 72 pc diameter for a distance $d=500$ pc. Between position angles 240° and 330°—measured from north over east centred on the pulsar position—the remnant extends further out to 5.2° (45 pc) along position angle 283°. The Vela pulsar 0833–45, which powers a small surrounding synchrotron nebula^{4,5}, is significantly offset by ~1.4° towards the west from the centre of the bright area, which suggests that the pulsar is not associated with the remnant². But within the new boundary, the pulsar's current position is offset from the centre of the best-matching circle by just 25 ± 5 arcmin, which is about one-tenth of the remnant's radius.

Figure 1 also shows at least six extended features, labelled A–F, well outside the boundary of the remnant. The properties of these features are listed in Table 1. A magnified image of each of the features is shown in Fig. 2. (The image of feature A in Fig. 2 was obtained from another pointed Rosat observation⁶.) The area containing feature D shows evidence for the superposition of two bow-shaped objects. Feature D shows a bright head like feature A, whereas D' further west looks like a diffuse arc. Five (B, C, D/D', F) of the protruding objects show a distinct 'boomerang' type structure which opens towards the SNR centre. The other two objects A and E look like truncated cones,

TABLE 1 Properties of the Vela SNR X-ray features

	X-ray feature							
	A _h	A _t	B	C	D _h	D _t	E	F
RA (2000) (h min s)	8 57 55	8 57 55	9 03 02	9 05 40	9 01 10	9 01 10	7 58 07	8 07 51
dec. (2000) (° ' ")	-41 49 22	-41 49 22	-43 33 05	-44 29 21	-45 31 41	-45 31 41	-44 38 00	-41 29 33
p.a. (°)	41.4	41.4	74.3	85.4	96.6	96.6	271.4	304.0
ε (°)	5.3	5.3	5.2	5.4	4.5	4.5	6.6	6.2
Ω (10 ⁻⁴ sr)	0.013	0.40	5.3	0.92	0.24	1.1	3.8	1.0
β (°)	29 ± 4	29 ± 4	41 ± 5	ND	41 ± 4	41 ± 4	50 ± 8	ND
F_X (10 ⁻¹² erg cm ⁻² s ⁻¹)	1.7	3.0	44.9	5.2	50.0	66.0	19.7	4.5
kT (keV)	0.59 ± 0.12	0.60 ± 0.15	0.39 ± 0.12	ND	0.87 ± 0.13	0.87 ± 0.16	0.28 ± 0.11	ND
N_H (10 ²¹ cm ⁻²)	0.54 ± 0.21	0.40 ± 0.12	0.24 ± 0.19	ND	0.29 ± 0.2	0.29 ± 0.2	0.48 ± 0.14	ND
Ma	4.0 ± 0.5	4.0 ± 0.5	2.8 ± 0.3	ND	2.8 ± 0.3	2.8 ± 0.3	2.4 ± 0.3	ND
v (km s ⁻¹)	650 ± 110	650 ± 110	450 ± 70	ND	450 ± 60	450 ± 60	390 ± 60	ND
kT_{exp} (keV)	0.60 ± 0.15	0.60 ± 0.15	0.34 ± 0.07	ND	0.34 ± 0.07	0.34 ± 0.07	0.27 ± 0.08	ND
V (10 ⁵⁶ cm ³)*	0.064	6.2	420.0	63.0	5.6	49.0	221.0	62.0
EM (10 ⁵⁶ cm ⁻³)*	0.063	0.061	0.67	0.077	0.78	0.95	3.4	0.067
n_H (cm ⁻³)*	0.89	0.089	0.036	0.032	0.37	0.12	0.11	0.030
L_X (10 ³² erg s ⁻¹)*	0.51	0.90	13.4	1.6	15.0	20.0	5.9	1.3

Physical data have been obtained by fits to the Rosat PSPC spectra assuming emission from an optically thin thermal plasma of standard cosmic abundances. Objects A and D show distinct surface-brightness enhancements close to the front, which have been analysed separately for their spectral characteristics; subscript h and t indicate head and tail, respectively. The spectra are fitted acceptably by models with at least two components of different temperatures. Only the high temperature is shown as it is most probably the indicator of the velocity of the object. Symbols used: RA (2000), right ascension; dec. (2000), declination; p.a., position angle measured from north over east; ε , angular distance from Vela pulsar; Ω , solid angle of object; β , cone opening angle; F_X , measured X-ray flux; kT , measured temperature $\times$ Boltzmann constant; N_H , total equivalent hydrogen absorption column density; Ma, Mach number; v , velocity of feature; kT_{exp} , expected temperature $\times$ Boltzmann constant; V , volume; EM = $\int n_e n_H dV$, emission measure; L_X , X-ray luminosity; n_H , hydrogen number density; n_e , electron number density; ND, not determined.

* These quantities are given for a distance of $d = 500$ pc. They scale as follows: EM $\propto d^2$, $V \propto d^3$, $L_X \propto d^2$ and $n_H \propto d^{-1/2}$.

which also open towards the remnant's centre. Objects A and E extend from the general SNR boundary by 1.2° and 2.4°, or for $d = 500$ pc, by ~ 10 pc and ~ 22 pc, respectively. The symmetry axes of these six structures intersect each other close to the remnant's geometric centre. The intersection of their symmetry axes with the known proper-motion vector of the pulsar⁷ of $(\mu_\alpha, \mu_\delta) = (-0.040 \pm 0.004, 0.028 \pm 0.002)$ arcsec yr⁻¹, gives the objects' origin at (14.9 ± 7.2) arcmin away from the present pulsar position along the proper-motion axis towards the southeast. The proximity of the remnant's geometric centre and the origin of the six protruding objects to the pulsar position strongly favours their common origin. Furthermore, the angular offset of the objects' origin divided by the proper motion velocity of 0.049 arcsec yr⁻¹ gives an independently determined age of the Vela SNR of $\tau = 18,000 \pm 9,000$ yr, assuming the objects' origin marks the explosion site of the progenitor star. Within the error range this datum is consistent with the pulsar's spin-down age⁸ $\tau_s = 11,400$ yr. However, the mean of 18,000 yr and the even larger offset of the centre of the best-matching circle inscribing the SNR periphery of 25 ± 5 arcmin, which corresponds to an age of $31,000 \pm 6,000$ yr, leave the possibility that the pulsar and the SNR are older than τ_s with an upper limit of 27,000 yr.

We suggest that the X-ray emission associated with the protruding objects is produced by shock-heating of the ambient medium by supersonic motion of the objects. This is strongly supported by the recent observation⁹ of radio emission from the leading edge of object A. Four of the protruding objects are trailed by wakes of X-ray emission, which we propose to be Mach cones, extending back to the blast-wave front of the Vela SNR. The Mach number Ma is related to the opening angle β of the cone by $Ma = (\sin \beta/2)^{-1}$. Mach numbers between 2.4 and 4.0 are found (Table 1). These low Mach numbers imply that the temperature of the ambient medium through which the objects are moving must be high, close to X-ray temperatures. Outside the general Vela SNR boundary, we have found in the Rosat all-sky survey data such an excess emission of almost constant surface brightness in a circular area with a diameter of 20° and the centre within the Vela SNR boundary. The analysis of the Rosat PSPC spectrum shows the temperature of this region to be 1.2 ± 0.2 K.

Given Ma and T_{amb} , we can determine the current velocity v and the expected temperature T_{exp} of each of the objects. T_{amb} is equivalent to a sound speed $c_s = 162 \pm 17$ km s⁻¹ in the ambient medium, using an adiabatic index of $\gamma = 5/3$. With the definition $Ma = v/c_s$, v is between 390 ± 60 and 650 ± 110 km s⁻¹. These

velocities are low compared with the mean velocity v_{mean} the objects had to have to reach their current angular distance ε from the pulsar, which is $v_{mean} = \varepsilon d/\tau$. For $d/\tau_s = 500$ pc/11,400 yr, $v_{mean} = 4,000$ –5,000 km s⁻¹, so that the objects seem to have been decelerated by a factor of ~ 10 . We note that v_{mean} for the blast wave is 3,200 km s⁻¹ with $\varepsilon = 4.15^\circ$. If the Vela SNR is in the adiabatic stage, the current velocity of the blast-wave front is expected to be $0.4v_{mean} = 1,280$ km s⁻¹. This

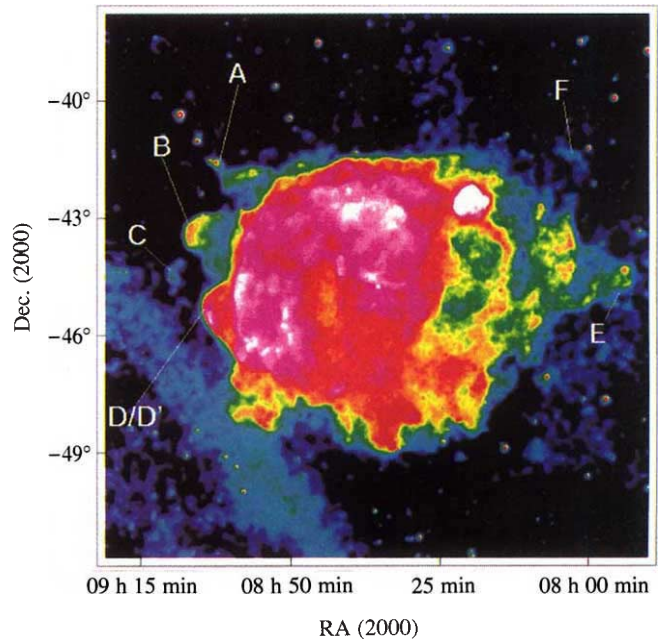


FIG. 1 Rosat all-sky survey image (0.1–2.4 keV) of the Vela supernova remnant. North is up and east is left, the angular resolution is 1 arcmin half-power radius, and the mean exposure is 993 s. The Vela pulsar 0833–45 is located at right ascension (RA) 8 h 35 min 20.7 s, declination (dec.) $-45^\circ 10' 35.7''$. Close to the northwestern corner at RA 8 h 20 min, dec. $-42^\circ 30'$ the bright SNR Puppis A appears, which lies behind the Vela SNR¹⁹. Surface brightness is colour-coded, increasing in the order light blue, yellow, red, white. Light blue to white represents a factor of 500. The blue stripe in the lower left-hand corner is due to unremoved scattered solar X-rays. Six objects outside the remnant's boundary are found, labelled A–F. Feature D/D' consists of two different objects D and D' which partially overlap along the line of sight.

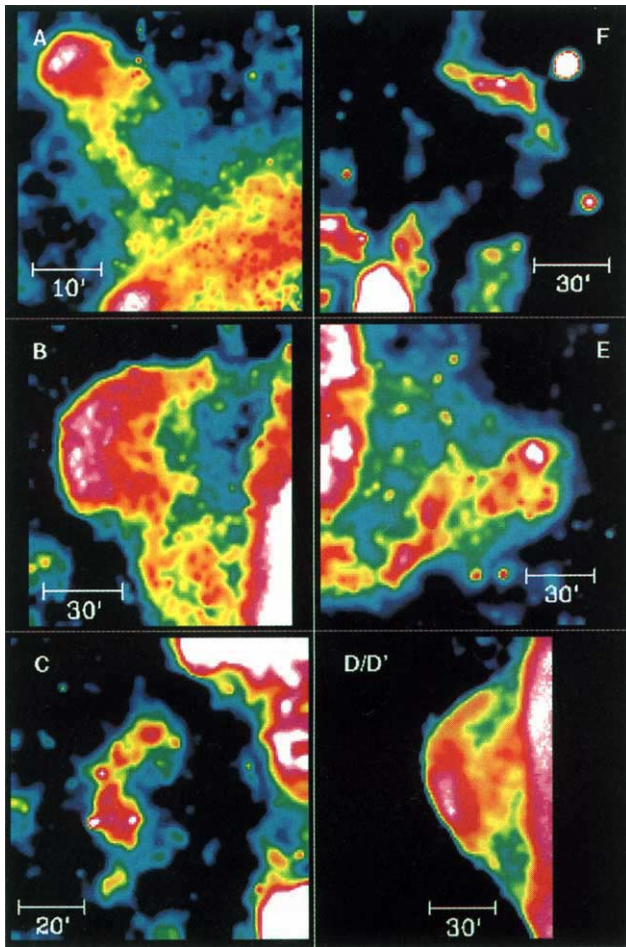


FIG. 2 Magnified X-ray images of the protruding objects A–F of Fig. 1. North is up and east is left in all the images.

is, however, at variance with the temperatures we observe from the Vela SNR rim, which require velocities lower by about a factor of 2, indicating that d/τ is lower than the standard value by the same factor. In this case the deceleration of the protruding objects has been less dramatic. A lower value of d/τ suggests that the Vela SNR is either older than 11,400 yr or closer than 500 pc, or both. With $\tau = 18,000 \pm 9,000$ yr, as determined above, $d = (400 \pm 200)$ pc.

The temperature T_{exp} expected for the objects by shock-heating is determined by the relation¹⁰

$$\frac{T_{\text{exp}}}{T_{\text{amb}}} = \frac{[2\gamma\text{Ma}^2 - (\gamma - 1)][(\gamma - 1)\text{Ma}^2 + 2]}{(\gamma + 1)^2\text{Ma}^2}$$

As can be seen from Table 1, kT_{exp} is between 0.27 and 0.60 keV. A comparison of kT_{exp} , inferred primarily from geometry, with kT , determined from measured spectra, shows very good agreement for each of the objects, which strongly supports the view that we see shock-heated gas confined in Mach cones. Object D, both head and tail, shows a significant discrepancy between $kT = 0.87 \pm 0.13$ keV and $kT_{\text{exp}} = 0.34 \pm 0.07$, which we propose is due to a projection effect. If object D is not moving in the plane of the sky but is moving out of it by an angle ϕ , Ma appears increased by a factor $1/\cos \phi$ and ε reduced by the same amount. With $kT = 0.87 \pm 0.13$ keV, Ma deprojected is 4.9 ± 0.7 , which gives $\beta = (24 \pm 4)^\circ$, similar to object A, and $\phi = (55 \pm 8)^\circ$; the true angular distance from the pulsar is then $(7.8 \pm 1.2)^\circ$, which is similar to that of object E.

With the spectral data of the objects available, a coarse estimate of the mass M of each object can be derived by equating the loss of kinetic energy $\Delta M v^2/2$ with the total thermal energy

content $n_{\text{H}} V k T$, including the wake contribution. Here, n_{H} is the hydrogen number density inside the emitting volume V . Using the currently observed velocity for v , ΔM is in the range 0.01–0.5 $M_{\odot}$, which is apparently a lower limit for M . The estimate for M scales with $d^{5/2}$, and a matter density filling factor of unity for V has been assumed.

We now mention two explanations for the origin of the protruding objects. (1) They could be due to interstellar clouds which have been accelerated at early times by the Vela blast wave to comparable speed. However, it is doubtful that clouds of the size and mass observed can survive such an encounter¹¹. (2) The objects might represent blobs of matter formed in the collapse of the progenitor star, and expelled in the subsequent supernova explosion. They travel largely unaffected through the hot, tenuous, post-shock plasma behind the blast wave and pass its front at a late stage, when the blast wave has significantly decelerated. Such a model—in which the explosion of some supernovae might more resemble a splinter bomb than a pressure bomb thus giving rise to long-lived confined explosion fragments—has been suggested by Kundt¹². Recent two-dimensional hydrodynamical calculations of supernova explosions of massive stars reveal the formation of Rayleigh–Taylor instabilities in the outer layers of the progenitor star near the H/He interface and the He/CO interface^{13,14} as well as convective instabilities close to the mantle of the nascent neutron star^{15,16}. Highly heterogeneous clumps of matter ('mushrooms') are formed by these instabilities.

The fact that we have observed objects in an SNR associated with a neutron star lends support to the presence of the types of instabilities mentioned above in type Ib or type II supernova explosions of massive stars. Whether we observe the effects which went on in the outer or deep inner layers remains to be seen. If, on the other hand, the creation of such fragments is bound to the birth of a neutron star, their occurrence might be as frequent as (or as rare as) that of neutron stars in SNRs, and the Vela SNR is one of these rare cases. In turn, the discovery of 'shrapnel' around other SNRs might point to the existence of a hidden, hitherto unknown neutron star.

The Rosat X-ray observations of the Vela SNR region present, for the first time, evidence for supernova explosion fragments coasting in front of the blast-wave boundary of a moderately old SNR. More detailed spectroscopic observations are required to deduce the physical conditions in the fragments and possibly to assess elemental abundances.

The observation of Mach cones imply that the Vela supernova exploded in a bubble of hot tenuous gas. The temperature of the ambient medium is as high as expected for old SNRs. It is well known that the Vela SNR lies in the direction of the huge Gum Nebula¹⁷—one of the most extended H α emission regions in the Milky Way. The origin and excitation of the Gum Nebula, however, have been discussed in terms of either an H II region excited by stellar ultraviolet radiation, or an old SNR¹⁸ (or a mixture of both). Our finding of hot gas inside the H II shell supports the SNR hypothesis. $\square$

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A radio-emitting X-ray ‘bullet’ ejected by the Vela supernova

Richard Strom*, Helen M. Johnston†, Frank Verbunt‡ & Bernd Aschenbach‡

* Netherlands Foundation for Research in Astronomy, Radiosterrenwacht, PO Box 2, NL-7990 AA Dwingeloo, The Netherlands

† Astronomical Institute, PO Box 80000, NL-3508 TA Utrecht, The Netherlands

‡ Max-Planck-Institut für extraterrestrische Physik, D-85748, Garching bei München, Germany

A MASSIVE star that explodes as a supernova produces an expanding remnant—a shell of gas—and leaves behind a neutron star. It has been suggested that instabilities during these explosions may create high-density clumps of ejecta^{1,2}, but such fragments have never been found in a remnant containing a pulsar. Here we present X-ray and radio observations of a feature outside the shock-wave boundary of the Vela supernova remnant (associated with the pulsar PSR0833–45), which appears to be an ejected fragment, with a wake³. The feature, which has travelled nearly 50 pc from the site of PSR0833–45 but is only one parsec across, is a clear-cut case of a ‘bullet’ of ejecta moving supersonically through the surrounding medium. The radio observations show non-thermal emission along the leading edge of the X-ray feature, indicating particle acceleration at the fragment’s shock front.

The Vela supernova remnant, G263.9–3.3, is a large (60 pc), nearby⁴ (500 pc) radio⁵ and X-ray^{6,7} shell, with intricate optical filaments⁸. Probable association with the central pulsar PSR0833–45 establishes its age⁹ at 11,000 yr. Observations of Vela^{3,7} in the Rosat all-sky survey show that the outer shell has a diameter of 7.3°, with several triangular protrusions extending up to 40% beyond its outer edge. X-ray knot ‘A’ (ref. 3) outside the northeastern rim of the supernova remnant (SNR), and its trailing emission, were discovered serendipitously in Rosat data obtained in a 8,256 s position-sensitive proportional counter (PSPC) observation¹⁰ of the cataclysmic variable CU Vel made at the end of May 1992. These data are presented and discussed here.

In addition to a number of point sources (including CU Vel), extended emission is seen in a roughly triangular-shaped feature which abuts the southwestern edge of the circular field of view (Fig. 1). This triangle of emission, brighter along its base, terminates at the northeastern apex in a prominent knot, or bullet, lying 5.3° (46 pc) from PSR0833–45. The knot has two peaks about ten times brighter than the bridge of emission which joins them to the southwestern rim. Although the leading edge of the knot may be unresolved, it is clearly extended, more so in the transverse direction, with dimensions of 7 × 4 arcmin in position angle 135°.

We obtained radio images of the region with the Very Large Array (VLA) at 6 cm (C band) and 21 cm (L band) in 1993 to look for evidence of particle acceleration. In the final maps, three point sources are visible at both wavelengths, together with a faint arc of emission. The total flux density of the arc is 4.3 mJy at C band, 17.0 mJy at L band, for a spectral index (ν^α) of $\alpha = -1.1$. The three compact sources have flux densities of 80, 8 and 4 mJy at C band, and spectral indices of -0.7 , -0.7 and -1.3 ,

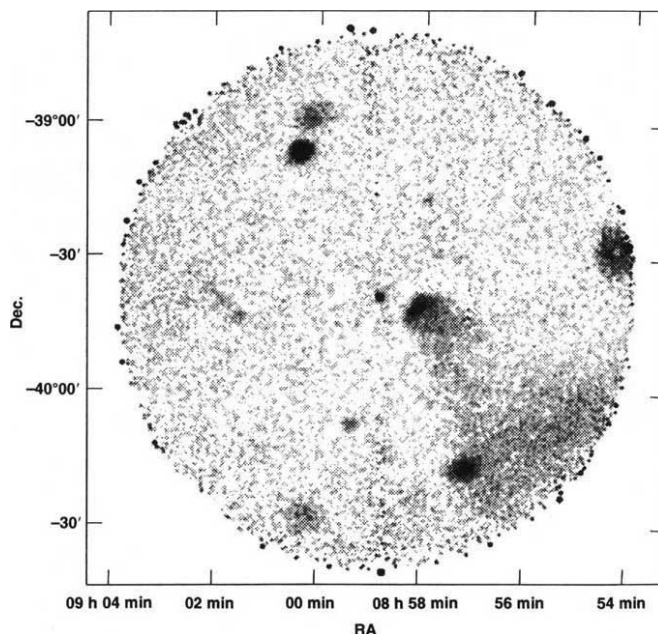


FIG. 1 The 0.1–2.2 keV X-ray emission from the entire field centred on CU Vel, as imaged by the Rosat PSPC, showing the extended knot 10 arcmin west of CU Vel, with a weak bridge trailing off to the lower right. (RA, right ascension; Dec., declination.) The bright source embedded in the bridge is a (non-related) flare star, showing the increase of size of the point-spread function towards the edge of the field. The extended emission in the lower right-hand corner is part of the northeastern rim of the Vela remnant. The data have been analysed in a standard way using the Extended Scientific Analysis System²¹ (EXSAS). The photons were binned into a single image with 15-arcsec pixels, which was flat-fielded by the exposure map and smoothed using a 25-arcsec gaussian (the resolution at the field centre).

respectively. The non-thermal ($\alpha < 0$) nature of all the emission means that it has almost certainly been produced by the synchrotron mechanism. The structural similarity of the maps gives us confidence that the arc of emission is genuine, despite its relative weakness.

Figure 2 shows the L-band radio map superimposed on the X-ray image. Radio emission is seen only around the periphery of the bright X-ray blob. The weak radio arc abuts the leading edge of the blob, with its intensity increasing where it wraps around the northwestern and southeastern sides. The strongest of the three compact radio sources lies southwest of (behind if motion is to the northeast) the blob, centred on the extended X-ray triangle with possible adjacent, faint radio emission. The two weaker radio components are immersed in extended emission where the arc bends around the southeast side of the knot. All three radio components are smaller than 15 arcsec. Although these compact sources could just be background objects, it seems unlikely that the three strongest peaks at a wavelength of 21 cm would all coincide by chance with extended emission associated with the knot. High-resolution studies of their morphology will probably be required to settle the issue. (Limited optical imaging, and examination of existing plate material, has failed to reveal emission which might be associated with either the X-ray or radio components.)

We fit a Raymond–Smith thermal emission model¹¹ to the Rosat spectrum to determine the energy kT (where T is temperature), volume emissivity, ϵ , and the column density of absorbing material, N_H . From the volume emissivity and assuming the triangular feature is rotationally symmetric about its major axis, that is along position angle 45°, we estimate average particle densities, n , in the emitting volumes, V . Parameters were calculated for the bright knot and trailing emission (tail) separately, and the values are given in Table 1. The similarity of both N_H

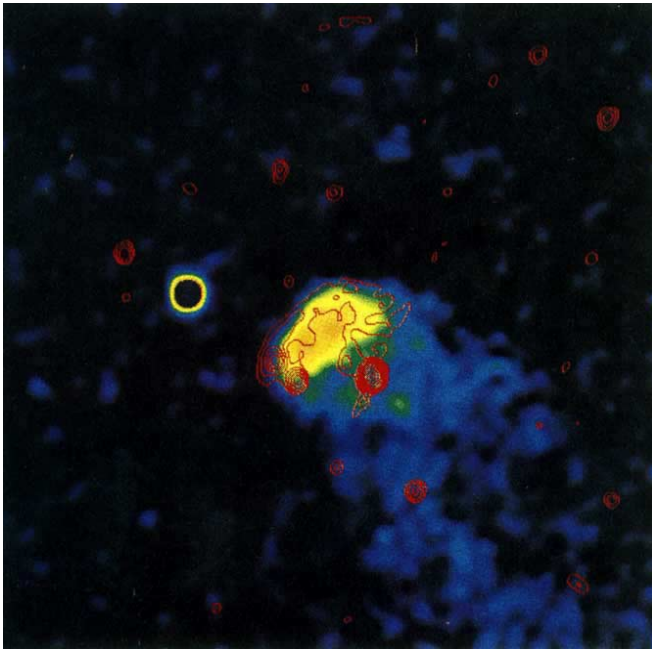


FIG. 2 The X-ray brightness distribution (colour shading) with overlaid L-band radio contours (red). The field is 41.75 arcmin on each side, centred on right ascension 8 h 57 min 45.0 s, declination $-41^{\circ} 50' 00.0''$ (J2000). The VLA L-band data were obtained in the CnB configuration at the standard frequencies of 1,385.1 and 1,464.9 MHz. Contour levels are 0.32, 0.64, 1.28, ..., 40.96 mJy per beam. 50-MHz bandwidths were used at each frequency, and the resolution was ~ 13 arcsec. The data were calibrated in the normal way using the NRAO ALPS package²². The bright source near the centre of the field was then used to self-calibrate the uv -phases (visibilities). The final map shown here has a resolution of ~ 40 arcsec. It has been further processed using a standard CLEAN algorithm^{23,24}, attaining a noise level of 122 μ Jy per beam.

and kT with those of Vela itself⁶ further strengthens the association.

The emission trailing behind the bullet looks very much like a Mach cone with an opening angle of $\sim 30^{\circ} \pm 5^{\circ}$. (The increase in the instrumental point spread function towards the edge of the field should not affect this estimate of the opening angle.) Although it is somewhat irregular, there is a very clear indication that the tail is rather limb-brightened. We conclude that we are seeing thermal X-rays from a sheath of gas shocked and compressed in the Mach cone generated by the supersonic blob. The opening angle suggests a Mach number $Ma \approx 4$. We note that at its base, the Mach cone appears to brighten and flare. This somewhat blunter feature is similar in form to the other extensions along the eastern rim of Vela³.

TABLE 1 X-ray and radio properties of the 'bullet'

Property	X-ray cone		Radio arc
	Knot	Tail	
N_H (10^{20} cm^{-2})	1.04 ± 0.35	1.4 ± 0.8	—
kT (keV)	0.34 ± 0.02	0.27 ± 0.05	—
n (cm^{-3})	0.36	0.04	—
V (10^{54} cm^3)	18	660	1.1
ϵ ($10^{-12} \text{ erg cm}^{-3}$)	194	24.4	2.2*

Symbols used: N_H , column density of absorbing material; kT , energy; n , average particle density in emitting volumes (V); ϵ , volume emissivity. The errors given are formal errors of the fit, and do not include uncertainties in the calibration or the spectral model.

* Calculated assuming energy equipartition.

The knot has an average speed of $v_k \approx 4,100 \text{ km s}^{-1}$ if it originated in the supernova explosion 11,000 yr ago. The only plausible explanation for it and the other protrusions—high-speed objects with an origin close to the central pulsar—is that they are the result of fragmentation of the outer layers of the progenitor during the supernova³. A similar origin has been suggested for the fast-moving knots¹² in the much younger SNR Cassiopeia A. Its oxygen-rich fragments, moving at speeds of $5,000 \text{ km s}^{-1}$ and more, have a composition which suggests an origin in the outer layers, and core, of a massive star^{12,13}.

The fast-moving knots in Cas A appear to have suffered little or no deceleration since their expulsion from the progenitor outer layers: each knot's distance from the centre simply reflects its initial speed¹⁴. Vela being much older, one might wonder to what extent the knot has been decelerated by its interaction with the surrounding gas. If the knot has travelled a distance, r_k , since its expulsion t s ago, then its present speed is

$$v_k = \eta \frac{r_k}{t} = Ma \, c \quad (1)$$

where c is the speed of sound in the ambient medium and η expresses the amount of deceleration the blob has experienced ($0 < \eta \leq 1$). There are several indications that the shell has encountered enough material to put it in the adiabatic phase^{7,15}. In terms of the shell radius, r_s , we then have the usual Sedov–Taylor solution¹⁶ for the shock speed:

$$v_s = \frac{2}{5} \frac{r_s}{t} \quad (2)$$

Assuming that the knot was expelled during the supernova explosion and that both it and the shell have been expanding into the same ambient medium, we eliminate t between equations (1) and (2):

$$v_s = \frac{2}{5} \frac{r_s}{r_k} \frac{Ma \, c}{\eta} = 1.1 \frac{c}{\eta} \quad (3)$$

For a strong shock, we require $v_s \gg c$, so from equation (3) we must have $\eta \ll 1$. This means that $v_k \ll 4,500 \text{ km s}^{-1}$, that is, the knot has been substantially decelerated. (The argument presented above also holds for motion not in the plane of the sky: because of projection, Ma will increase, but as r_k is also foreshortened it increases by the same factor, leaving equation (3) unchanged.)

The deceleration found means that the knot has been interacting strongly with the ambient medium, and some of the energy dissipated has gone into particle acceleration along its periphery (Fig. 2). As is well known, diffusive particle acceleration occurs in strong shocks¹⁷, although it should be noted that in this case, it apparently takes place in one of modest Mach number ($Ma < 5$). On the basis of the minimum internal energy density from the radio arc, ϵ (Table 1), the implied magnetic field strength is $4 \mu\text{G}$. This estimate is nearly 100 times smaller than the thermal energy density of the knot, suggesting that if it is ram pressure confined (as concluded below), then the efficiency of particle acceleration is only about 1%. In the brightest peak, ϵ exceeds the thermal pressure, suggesting that if the peak is associated it is probably unconfined and transient.

As the knot ploughs through the interstellar medium, it experiences a ram pressure of ρv_k^2 . This is sufficient to confine it if¹⁸

$$\rho v_k^2 \approx \epsilon \quad (4)$$

The ratio of the ambient density, ρ , to the knot density can be estimated as ~ 0.02 from the ratio of the knot size to the distance it has travelled¹⁹. The knot density (Table 1) then suggests an ambient particle density of $\sim 0.01 \text{ cm}^{-3}$ (which is similar to the average density found for the X-ray tail, Table 1), and through equation (4) we then estimate $v_k \approx 1,000 \text{ km s}^{-1}$. This is roughly consistent with the limit, $v_k \ll 4,500 \text{ km s}^{-1}$, found above.

We conclude that the knot is probably confined by the ram pressure of the ambient medium. Its mass ($\sim 0.005 M_{\odot}$) is comparable to the average value estimated²⁰ for the fast-moving knots in Cas A ($10^{-3} M_{\odot}$), and we suggest that the two phenomena are related. Fragments of a massive progenitor can be expelled at speeds of the order of 10^4 km s^{-1} , possibly through instabilities whose existence is indicated by recent hydrodynamic calculations of supernova explosions^{1,2}. Under favourable circumstances such knots can travel distances of 50 pc and remain intact for over 10^4 yr . We presume that they have not been observed in other evolved SNRs because of the intrinsic faintness of their emission, combined with a soft X-ray spectrum which results in heavy interstellar absorption even at moderate distances.

The radio emission that we find indicates that particle acceleration occurs very close to the shock front, over a distance no larger than $2 \times 10^{17} \text{ cm}$. The shock acceleration appears to be greatest along the lateral edges of the knot rather than at its leading stagnation point, possibly because higher densities there and internally may quench the acceleration process (A. Achterberg, personal communication). The Vela bullet constitutes an unusual astrophysical laboratory for testing magnetohydrodynamic calculations of fluid motion and particle acceleration at a shock front of modest Mach number. As such it merits further investigation. $\square$

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Chemical and thermal response of Jupiter's atmosphere following the impact of comet Shoemaker–Levy 9

E. Lellouch*, G. Paubert†, R. Moreno†, M. C. Festou‡, B. Bézard*, D. Bockelée-Morvan*, P. Colom*, J. Crovisier*, T. Encrenaz*, D. Gautier*, A. Marten*, D. Despois§, D. F. Strobel|| & A. Sievers†

* Observatoire de Paris-Meudon, F-92195 Meudon, France

† IRAM, Granada, Spain

‡ Observatoire Midi-Pyrénées, F-31400 Toulouse, France

§ Observatoire de Bordeaux, F-33270 Floirac, France

|| The Johns Hopkins University, Baltimore, Maryland 21218, USA

In July 1994, the collisions of the fragments of comet Shoemaker–Levy 9 with Jupiter resulted in dramatic changes in the planet's atmosphere. Observations of the events suggest that the composition and thermal properties of the atmosphere were considerably modified at the impact sites, with the changes persisting for times lasting from minutes to weeks (see, for example, refs 1–4). Here we report observations of the impact sites at millimetre wavelengths, which reveal strong emission lines associated with carbon monoxide, carbonyl sulphide and carbon monosulphide. The abundance of carbon monoxide in the jovian atmosphere is normally very low⁵; carbonyl sulphide and carbon monosulphide, on the other hand, have not hitherto been detected. We find that the largest fragments (G and K) each produced approximately 10^{14} g of carbon monoxide, $3 \times 10^{12} \text{ g}$ of carbonyl sulphide and $3 \times 10^{11} \text{ g}$ of carbon monosulphide, most probably by shock-induced chemical reactions⁶. Our observations also place firm constraints on the thermal response of Jupiter's stratosphere to the impacts.

Observations of impact sites were conducted at the Institut de Radio Astronomie Millimétrique (IRAM) 30-m telescope at Pico Veleta, Spain, on 17–28 July 1994. We searched for rotational lines of several molecular and ionic species that could

plausibly be injected into, or generated in, Jupiter's atmosphere by the impacts. The telescope beam (12 arcsec full-width at half-maximum (FWHM) at 230 GHz) did not resolve the sites and usually encompassed several sites after the first observing days.

Three compounds were detected (Figs 1 and 2): CO at 230.538 GHz (the $J=2-1$ line) on various dates and impact sites C, E, G, H, K and L, and complexes G+Q and G+Q+R+S; CS at 244.935 GHz ($J=5-4$) on impact sites K and L, and complexes G+Q+R+S and K+W on 21 July and later; and OCS at 218.903 GHz ($J=18-17$) on 22 July at impact complex K+W. Carbon monoxide was also observed at 115.271 GHz ($J=1-0$) on the G+Q+R+S complex on 23 July. ("Site C" refers to the site produced by the impact of fragment C; "complex" indicates that several sites are contained in the telescope beam.) CO ($2-1$) was also detected on 23 July by the Swedish ESO submillimetre telescope (SEST)⁷. As expected, the lines moved in frequency as Jupiter rotated, demonstrating that they originated from the impact regions. Upper limits were obtained on the abundance of non-observed CO^+ , CN, H_2CO , H_2S , SO_2 , CH_3OH and HC_3N (Table 1).

Monitoring of the CO and CS lines was performed for a few impact sites, particularly the G+Q+R+S complex. Until 25 July, all lines appeared as narrow emission features ($\sim 2.5 \text{ MHz}$

TABLE 1 Abundances of CO, CS and OCS, and upper limits on other minor compounds

Molecule	Frequency (GHz)	Date of obs.	Site(s) or complex	Mass in beam* (g)
CO	230.538	18 July	G	1×10^{14}
CO	230.538	21–28 July	G+Q+R+S	2×10^{14}
CS	244.935	21, 28 July	G+Q+R+S	6×10^{11}
CS	244.935	22 July	K+W	5×10^{11}
OCS	218.903	22 July	K+W	5×10^{12}
CO^+	235.380	18 July	G, H	$\leq 5 \times 10^{10\dagger}$
H_2S	216.710	19 July	E, G, H	$\leq 3 \times 10^{12\dagger}$
SO_2	216.643	19 July	E, G, H	$\leq 3 \times 10^{12\dagger}$
CH_3OH	218.440	20 July	K, G+Q	$\leq 4 \times 10^{12\dagger}$
HC_3N	218.325	20 July	K, G+Q	$\leq 2 \times 10^{10\dagger}$
H_2CO	218.760	22 July	G+Q+R+S	$\leq 2 \times 10^{12\dagger}$
CN	226.762	25 July	G+Q+R+S	$\leq 5 \times 10^{11\dagger}$

* Sum of beam-weighted masses in impact sites present in the beam.

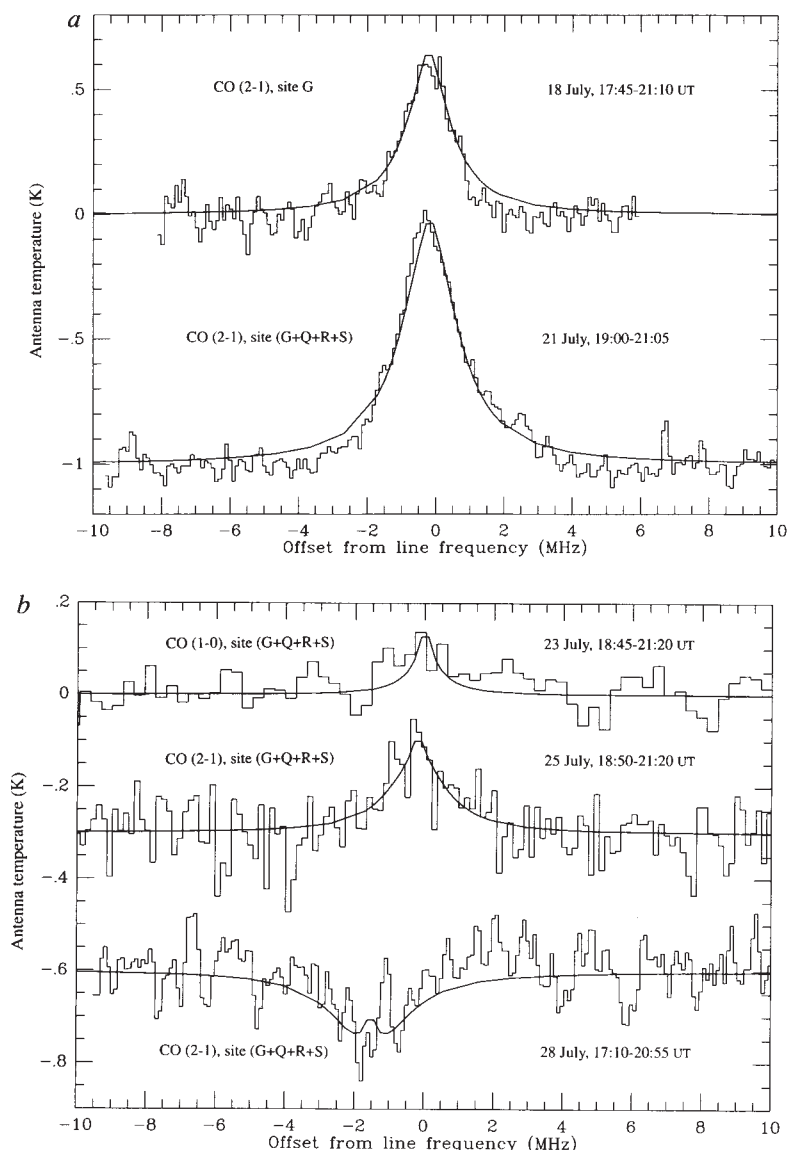
† 2σ upper limit, rescaled to G impact.

FIG. 1 CO emission lines from sites G and G+Q+R+S (histograms), along with best model fits (solid lines). *a*, CO (2–1) line (230.538 GHz), observed on site G (18 July) and G+Q+R+S complex (21 July). Retrieved parameters are: $\Delta T = 30$ K, $q_{\text{CO}} = 4 \times 10^{-5}$ (uniform above the 0.3-mbar level) for site G; $\Delta T = 28$ K, $q_{\text{CO}} = 5 \times 10^{-5}$ (same distribution) for complex G+Q+R+S. Optical depths at line centre are 1.9 and 2.5, respectively, consistent with a moderately saturated lineshape. The probed region extends up to ~ 0.1 mbar. Similar fits can be obtained by assuming increased temperature gradients in the 10–0.1 mbar region. In this case, lower CO mixing ratios are derived ($(0.5\text{--}2) \times 10^{-5}$), but down to deeper pressure levels (0.7–1.5 mbar), so that, within a factor of 2, the total inferred CO mass is unchanged. *b*, Evolution of the CO lines on complex G+Q+R+S on 23 July (CO (1–0), 115.271 GHz), 25 July (CO (2–1)) and 28 July (CO (2–1)). All models assume $q_{\text{CO}} = 5 \times 10^{-5}$ above 0.3 mbar. Retrieved ΔT at $p \leq 2$ mbar are +10, –3 and –15 K for 23, 25 and 28 July respectively. Models were shifted in frequency when necessary to match the observed line central frequencies. Multisite modelling and simultaneous fits to the SEST observations will be necessary to provide fully satisfactory solutions.

METHODS. For all observations presented here, a “balanced position switch” method was adopted, in which the telescope was alternately pointed to an impact site, then to the position diametrically opposed on Jupiter’s disk. Velocity tracking was performed by recording 1-min scans which were co-added after appropriate velocity corrections. For line calibration, an emitting region of diameter 2.5 arcsec ($\approx 9,500$ km) is assumed for site G on 18 July. For the G+Q+R+S complex on 21 July and later, some regions of which are underweighted by the 12-arcsec beam, we assume a total effective 3.2-arcsec (12,000-km) region at CO (2–1) and a 4-arcsec (15,000-km) region at CO (1–0). Velocity corrections for the G+Q+R+S complex refer to the R site. All models in this work include continuum absorption by H_2 , He and NH_3 and an absorbing layer at 300 mbar (transmission 0.95) to match the disk-averaged brightness temperature $T_{\text{B}} \approx 170$ K near 1.3-mm wavelengths²⁰. (Centimetre²¹ and 13–20 μm (refs 2, 3) observations indicate that the tropospheric temperatures did not change by more than 2 K). An airmass of 2 in Jupiter’s atmosphere is adopted. Models further assume a uniform temperature difference (ΔT) relative to pre-impact conditions at $p \leq 2$ mbar.

(3 km s^{–1}) FWHM for CO (2–1), 3 MHz (4 km s^{–1}) for CS (5–4)) with contrasts (in antenna temperature (T_{A}^*)) between 0.1 and 1 K. Linewidths remained fairly constant, although some slight increase was noticed as more sites accumulated in the beam. Line intensities, conveniently described by their integrated area, generally declined over a few days (Fig. 1). In some cases however (sites H, K and G+Q), maximum intensities were not reached immediately after impact but rather 1–2 days later. On 28 July, CO and CS were detected in absorption (with possibly, in the case of CO, a narrower emission core). We interpret the initial intensity increase as being caused by the expansion of the impact sites, whereas the subsequent decrease and the switch over to the absorption regime are primarily due to the cooling of the gas. Line positions for the G+Q+R+S complex show that a large fraction of the emission comes from the R site, although some frequency mismatches (up to ~ 1.5 MHz) occur, presumably indicative of a multisite emission.

The observed transitions correspond to thermal absorption/emission of Jupiter’s atmosphere as modified by the impacts. The linewidths must reflect a combination of pressure (Lorentz) broadening (thermal Doppler broadening would indicate an implausible temperature of $\sim 5,000$ K hours or days after the impacts) and velocity smearing. Pressure broadening would suggest that the molecules are present near the ~ 0.5 -mbar level in

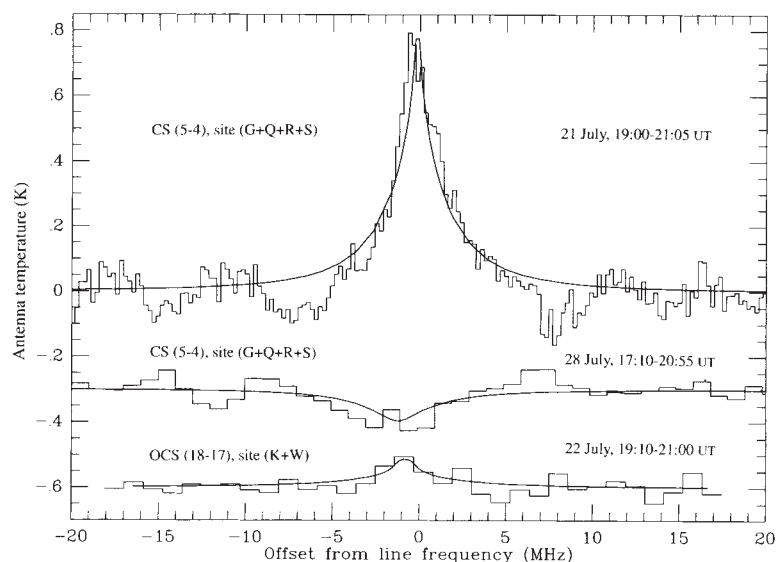


the atmosphere. Velocity smearing inside the impact sites must occur to some degree, but is probably not the dominant effect because (1) the lines are only slightly narrower near the limb, (2) the CO 245-GHz and the CO 230-GHz lines do not have the same widths, and (3) the two CO lines at 115 and 230 GHz have similar widths in frequency, not in velocity. Yet, the slight linewidth increase with time probably reflects the increasing contribution of the velocity dispersion within the sites. We defer the detailed study of the linewidths and line positions to future work and here assume the lines to be solely pressure-broadened.

Conversion from antenna to brightness temperatures requires knowledge of the size of the emitting region. We assumed that the ammonia 10- μm stratospheric emission, observed⁸ on site K at 21 h, 5 d and 10 d after impact, can be used as an indicator of the spatial distribution of the emissions we observe. Emitting-region diameters in the range 2.5–4 arcsec were thus assumed (Figs 1 and 2). The strongest CO and CS emission lines calibrated in this manner (18 and 21 July) correspond to brightness temperature contrast $\Delta T_{\text{B}} \approx 38$ K, whereas the absorption lines seen on 28 July have $\Delta T_{\text{B}} \approx -8$ K.

Observations were modelled by means of a radiative transfer code⁹, in which the minor species were assumed to be uniformly mixed above a pressure level essentially determined by the linewidths. For the geometry of our averaged observations, the con-

FIG. 2 CS (5–4) line (244.935 GHz) on complex G+Q+R+S, observed on 21 and 28 July (histograms), and OCS (18–17) (218.903 GHz) line on site K+W (22 July), along with best model fits (solid lines). Note that these two molecules were detected as soon as we attempted to observe them. For the CS lines, velocity corrections refer to the R site. Models assume the same emitting region as for CO (2–1), and make use of the ΔT s from the corresponding CO (2–1) lines, that is, $\Delta T = +28$ and -15 K on 21 and 28 July, respectively. The retrieved CS mixing ratio is 4.5×10^{-8} above the 0.7-mbar level. Optical depth at line centre is 0.75. For the OCS spectrum, taken ~ 10 h and 3 d after the W and K impacts, respectively, a 3.1-arcsec (11,500-km) emitting region and a temperature warming relative to pre-impact conditions of $\Delta T = 10$ K at $p \leq 2$ mbar are assumed. The retrieved OCS mixing ratio is 2×10^{-7} above the 1-mbar level (optical depth at line centre is 0.25). A CS spectrum taken simultaneously (not shown here) indicates a CS mixing ratio of 2.5×10^{-8} , that is, CS/OCS = 1/10 in mass.



tinuum T_B is ~ 160 K. The calibrated line contrasts imply that ~ 10 h after the large impacts, the stratosphere near the 0.5-mbar level had an excess temperature of at least $\Delta T = 25$ –30 K over its normal 170 K temperature¹⁰. Much more heating could be accommodated by the observed contrasts, but would probably be inconsistent with the 7.85- μ m CH_4 emission², which formally corresponds to $\Delta T \approx 3$ –4 K at ~ 20 mbar 10–20 h after impacts. Adopting a uniform $\Delta T = 30$ K at $p \leq 2$ mbar, the 18 July line (site G), implies a CO mixing ratio $q_{\text{CO}} = 4 \times 10^{-5}$ at $p \leq 0.3$ mbar, that is, a CO mass of $\sim 1 \times 10^{14}$ g. Good fits can also be obtained by assuming increased temperature gradients in the 10–0.1 mbar region, leading to the same total CO mass, within a factor of two. Obtaining more accurate values of T and q_{CO} will require that the pressure/velocity effects on the line shape be fully deconvolved.

The evolution of the CO lines on site G+Q+R+S was modelled assuming a constant CO distribution between 21 and 28 July. This is justified because (1) CO is chemically stable and its abundance not expected to increase after its shock formation (see below), (2) the vertical transport time is ~ 3 yr for typical stratospheric eddy diffusion coefficient $K = 10^5 \text{ cm}^2 \text{ s}^{-1}$ (ref. 11), (3) the lateral spreading of the visible spots over 1 week is much less than the extent of the beam. The total “effective” CO mass in the beam derived from these spectra is $\sim 2 \times 10^{14}$ g, and stratospheric temperatures differ from the “quiescent” profile by $\Delta T =$

+28, +10, -3 and -15 K on 21, 23, 25 and 28 July respectively, indicating that the thermal profile at 0.1–1 mbar did not simply relax to its pre-impact state but became colder ~ 4 d after impact (Fig. 3).

Modelling of the CS and OCS lines on the G+Q+R+S and K+W complexes (Fig. 2 and Table 1) indicates CS:OCS:CO mass ratios of 1:10:320. By scaling to the site G 18 July spectrum (assuming a similar composition for all fragments), we conclude that the largest fragments (G, K) generated $\sim 1 \times 10^{14}$ g of CO, 3×10^{12} g of OCS and 3×10^{11} g of CS. Upper limits of the abundances of other compounds, similarly rescaled, are given in Table 1.

Carbon monoxide has also been detected in various infrared observations^{12–14}, but abundance measurements are not yet available. The CO mixing ratio we derive, $\sim 50,000$ times larger than the normal Jupiter CO tropospheric abundance⁵, clearly implies that we are not observing jovian CO transported in the plume. Our inferred masses ($\sim 6 \times 10^{13}$ g oxygen and $\sim 2 \times 10^{12}$ g sulphur) and the associated O/S ratio (65 in volume) are consistent with the amount of O and S available in $\sim 10^{14}$ -g cometary fragments¹⁵. A definite answer regarding the origin of the oxygen and sulphur however requires comparison with other data sets. Hubble Space Telescope (HST) observations¹ of $\geq 10^{14}$ g S_2 favour a jovian origin for the sulphur and therefore penetration of the largest fragments down to at least the NH_4SH cloud near

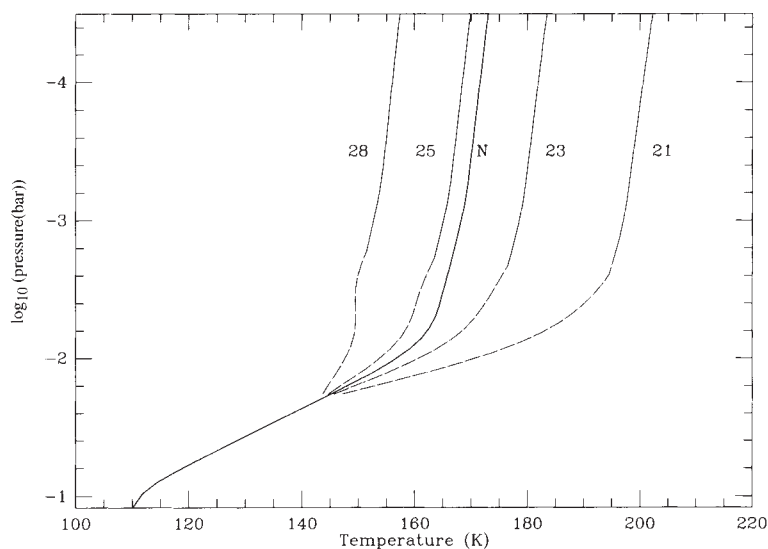


FIG. 3 Temperature profiles inferred for the G+Q+R+S complex on 21, 23, 25 and 28 July, compared to pre-impact profile⁹ (labelled N). Temperature information is restricted to the $\sim (0.1$ – $2)$ mbar range and the profiles are retrieved under the arbitrary assumption that they show a constant temperature shift with respect to the normal profile. Dashed lines indicate possible (but unconstrained) profiles below the 2-mbar level.

1.7 bar. Bjoraker *et al.*³ argue that their observations of abundant stratospheric water (with $\text{H}_2\text{O} \gg \text{CH}_4$) suggest that the water cloud near 5 bar (below which $\text{H}_2\text{O} \approx \text{CH}_4$) was not reached. If true, the oxygen seen in CO and H_2O must be of cometary origin. However, the high temperatures generated by the impacts ($>15,000$ K in the fireball¹⁶) are expected to dissociate most of the cometary and jovian material in the fireball. Shock chemistry⁶ seems to provide the most plausible way of (re-)forming molecular species. A recent model¹⁷ shows that shock chemistry takes place in two steps, first in the fireball, and then during plume re-entry at 0.01–1 mbar. For a 1-km-diameter cometary fragment with solar composition, $\sim 10^{14}$ g of CO (along with a similar amount of H_2O and a smaller mass of CO_2) and a few times 10^{12} g of OCS are predicted to form (mostly in the fireball phase for OCS, and in both phases for CO), essentially in agreement with our measurements. This model¹⁷, however, apparently predicts the formation of only small amounts of CS, consistent with the abundance seen in an HST (Faint Object Spectrograph) spectrum of site S taken 45 min after impact ($\sim 10^9$ g; ref. 1), but much less than the mass we infer. Shock chemistry is also expected to produce abundant CS_2 (actually seen by HST) from jovian gas. Photochemical modelling¹⁸ indicates that CS_2 undergoes photolysis into CS + S with a lifetime of ~ 1 week near 1 mbar. So whereas the HST spectra may have seen the first CS component produced in the fireball, our measurements may have detected the large amounts of CS built up from CS_2 photolysis.

Our measurements imply that Jupiter's mid-stratospheric thermal profile remained modified a long time after the impacts. In particular, an important cooling occurs at least over a timescale of a week, leading to temperatures colder than before

the impacts. Although the formation¹⁹ of a dark haze at the 1–200 mbar level must lead to increased heating in this region, it is clear that this effect is offset by unusual cooling. The injection into the stratosphere of considerable amounts of minor compounds, some of which radiate efficiently in the infrared and submillimetre ranges (H_2O , NH_3 , HCN and so on), might be the main reason for this long-lasting temperature modification. □

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Phase-contrast imaging of weakly absorbing materials using hard X-rays

T. J. Davis, D. Gao, T. E. Gureyev, A. W. Stevenson & S. W. Wilkins

CSIRO Division of Materials Science and Technology, Private Bag 33, Rosebank MDC, Clayton, Victoria 3169, Australia

IMAGING with hard X-rays is an important diagnostic tool in medicine, biology and materials science. Contact radiography and tomography using hard X-rays provide information on internal structures that cannot be obtained using other non-destructive methods. The image contrast results from variations in the X-ray absorption arising from density differences and variations in composition and thickness of the object. But although X-rays penetrate deeply into carbon-based compounds, such as soft biological tissue, polymers and carbon-fibre composites, there is little absorption and therefore poor image contrast. Here we describe a method for enhancing the contrast in hard X-ray images of weakly absorbing materials by resolving phase variations across the X-ray beam^{1–4}. The phase gradients are detected using diffraction from perfect silicon crystals. The diffraction properties of the crystal determine the ultimate spatial resolution in the image; we can readily obtain a resolution of a fraction of a millimetre. Our method shows dramatic contrast enhancement for weakly absorbing biological and inorganic materials, compared with conventional radiography using the same X-ray energy. We present both bright-field and dark-field phase-contrast images, and show evidence of contrast reversal. The method should have the clinical advantage of good contrast for low absorbed X-ray dose.

Phase-contrast imaging at optical wavelengths is an important technique for studying thin biological samples *in vivo* and without staining. Such imaging is achieved by interfering a reference wave with waves transmitted through the sample^{5,6}. The phase gradients in the transmitted beam, arising from refractive-index variations in the sample, lead to contrast in the image. Although this technique has been used for many years to discern features in weakly absorbing materials, the lack of lens systems for short wavelengths ($\lambda < 1$ nm) has prevented the development of analogous systems for hard X-rays. However, a phase gradient across a wavefront is equivalent to a change in the direction of propagation of the wave⁷. Any optical system which is sensitive to the direction of propagation of the X-ray is therefore capable of resolving phase gradients and can be used for phase-contrast imaging. For example, high-sensitivity angular analysers for monochromatic X-rays may be constructed from perfect crystals⁸, such as silicon, and are used routinely in the characterization of crystalline materials by X-ray diffraction⁹.

Figure 1 shows one possible method of X-ray phase-contrast imaging, in which two monolithic silicon crystals sharply define the X-ray wavefront and provide high angular sensitivity to the refracted X-rays. The first crystal acts as a monochromator on the X-ray beam and, because the crystal surface has been cut at an angle to the diffracting crystal planes, also spatially expands the beam and collimates it (asymmetric diffraction^{10,12}). This produces an almost-plane wavefront which passes through the sample. The second crystal, cut from a monolithic block of silicon, is an analyser that only diffracts plane waves propagating in a predetermined direction and redirects these to an X-ray detector or film. The refractive-index variations in the sample distort the wavefront from its planar state. The distortions are rejected by the analyser, resulting in contrast in the image associated with refractive-index variations in the sample^{1,4}. By changing the angle of the analyser relative to the first crystal, different planar sections of the distorted wavefront can be imaged, leading

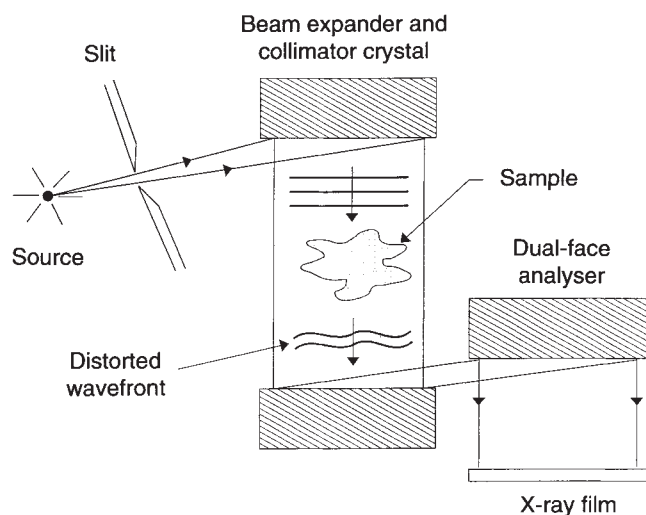


FIG. 1 One possible configuration for obtaining hard X-ray phase-contrast images using perfect crystals of silicon. The crystals are cut so that the surface planes are almost parallel to the Bragg planes for the diffracting X-rays, giving rise to asymmetric diffraction. The asymmetric diffraction from the first crystal expands the beam and collimates it, producing a plane wave which refracts on passing through the sample. The refraction across the wavefront depends on the density gradients in the sample. The analyser uses two asymmetric crystal faces cut from a monolithic block of silicon. The first face gives high angular sensitivity but reduces the beam cross-section. The second face expands the beam back to its original size for imaging. The analyser can be rotated to accept different parts of the refracted wave which alters the contrast in the image. For the analyser used in our experiments, the full-width at half-maximum of the analyser response curve is 1.0 arcsec. The spatial resolution in the images (Figs 2, 3 and 4) is limited by the divergence of the X-ray beam, which is determined by the size of our laboratory X-ray source.

to a series of images in which different features of the object have differing contrast (T.J.D., manuscript in preparation).

In a more formal sense, the propagation of X-rays through an X-ray-transparent medium is accompanied by a change in the phase of the wavefront relative to vacuum, as for the case of visible light passing through an optically transparent medium. The phase change for X-rays is related to the forward scattering from the electrons in the medium and in the short-wavelength limit is given by^{13,14}

$$\phi(x, y) = -r_e \lambda \int_M \rho(x, y, z) dz \quad (1)$$

where $\rho(x, y, z)$ is the electron density at point (x, y, z) , r_e is the classical electron radius, λ is the wavelength of the X-rays and the integration is over the path of the X-ray beam in the medium, M . It is assumed that the X-rays are propagating in the z -direction.

For a wave, ψ , initially propagating in the z direction with wavenumber k and suffering phase changes, $\phi(x)$, in the x -direction, the X-ray wave is given by

$$\psi(x, z) = \psi_0 e^{ikz + i\phi(x)} \quad (2)$$

and the wavevector in the direction of propagation is

$$\mathbf{k}'(x) = \frac{1}{i\psi(x, z)} \nabla \psi(x, z) = \frac{\partial \phi(x)}{\partial x} \mathbf{x} + k \mathbf{z} \quad (3)$$

For small phase gradients, the angle of propagation relative to the initial propagation direction is

$$\Delta\alpha \approx \frac{1}{k} \frac{\partial \phi(x)}{\partial x} \quad (4)$$

and corresponds to the angular offset of the analyser required to accept this directional component of the wavefront, leading to the possibility of contrast formation in an image recorded after the analyser crystal. The precise nature of the contrast depends *inter alia* on the convolution of the wave with the complex point-spread function of the analyser crystal (T.J.D., manuscript in preparation).

In the present case of 8.048-keV ($\lambda = 0.154$ nm) X-rays, 422 Bragg reflections from perfect-crystal silicon were used for both the monochromator and analyser. The beam emerging from the face of the monochromator was collimated to of the order of 0.8 arcsec, which is matched to the angular acceptance of the double-reflection analyser crystal. The beam emerging from the monochromator is also spatially expanded by a factor of 12 across the propagation direction due to the asymmetry effect.

The combined system of monochromator and analyser crystals acts non-dispersively; when the analyser is set at the exact Bragg condition for diffraction of the beam from the first crystal, all wavelengths diffracted by the first crystal are also at the correct Bragg angle for diffraction by the second crystal. Because the first and second faces of the analyser successively laterally compress and expand the beam by equal amounts, there is no net lateral magnification of the image.

Comparisons between radiography based on absorption-contrast and phase-contrast imaging show dramatic differences. To illustrate the technique—actually using a monolithic monochromator/analyser system⁴—we examined a leaf (*Eucalyptus melliodora*) (Fig. 2). In the case of a radiograph taken with the film in front of the analyser (Fig. 2a), some very weak absorption (negative) contrast of vein structure is seen. But in the case where the film is placed after the analyser, very strong positive contrast is seen (Fig. 2b). This latter image may be considered to be a dark-field phase-contrast image with a fixed offset of the analyser crystal; we believe, from relative intensities measured before and after the analyser, that strain was responsible for a slight offset (of the order of 1 arcsec) of the analyser relative to the monochromator.

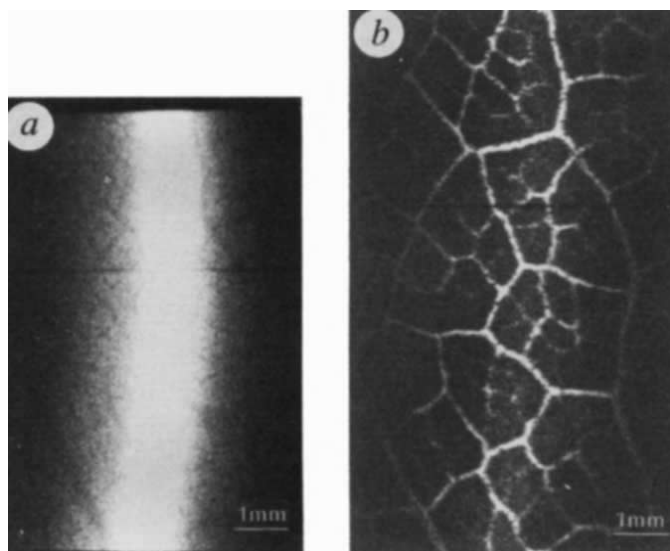
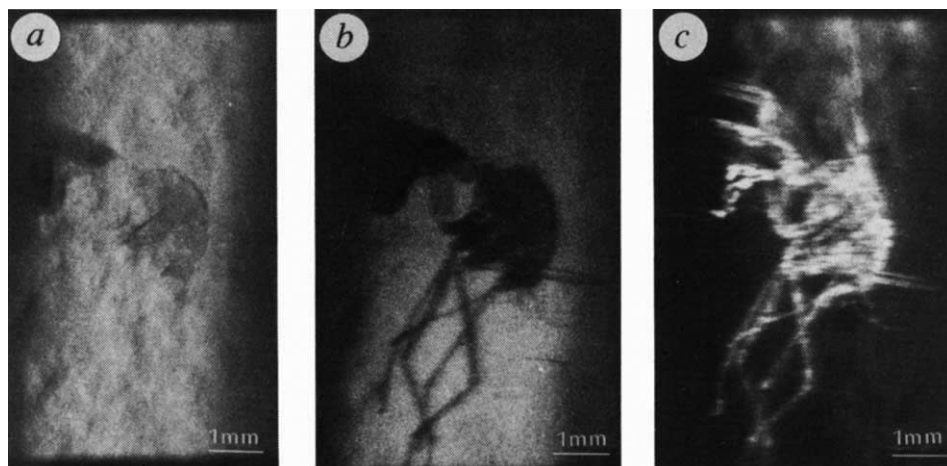


FIG. 2 A comparison between a radiograph (a) and a phase-contrast image (b) of a leaf (*Eucalyptus melliodora*) using a fixed-angle analyser crystal with offset estimated to be of the order of 1 arcsec. The phase-contrast image is magnified slightly after the analyser.

FIG. 3 Comparisons between a radiograph (a) and phase-contrast images (b, c) of a mosquito. a, Radiograph taken immediately in front of the analyser crystal. The non-uniformities in the background are due to imperfections in the first crystal. b, Phase-contrast image taken after the analyser which has been offset by 0.4 arcsec from the peak-reflection condition. c, Contrast reversal is seen in this phase-contrast image when compared with b. This results from the analyser rejecting that part of the plane wave which has missed the sample but accepting that part which has refracted through the sample by an appropriate angle. The analyser is offset by 1.6 arcsec. Note that the wings to the rear of the mosquito are beginning to show contrast.



Images of a mosquito placed between the two crystals of Fig. 1 are shown in Fig. 3. There are striking differences between the conventional radiograph (Fig. 3a) and the phase-contrast exposures taken at different analyser offset angles (Fig. 3b, c). At low offset angles, the image of the mosquito appears dark on a bright background because the analyser rejects the X-rays passing through the sample, most of which are refracted away from the direct beam. At higher offset angles the direct beam is rejected while some of the refracted beams are accepted, leading to a reversal of image contrast.

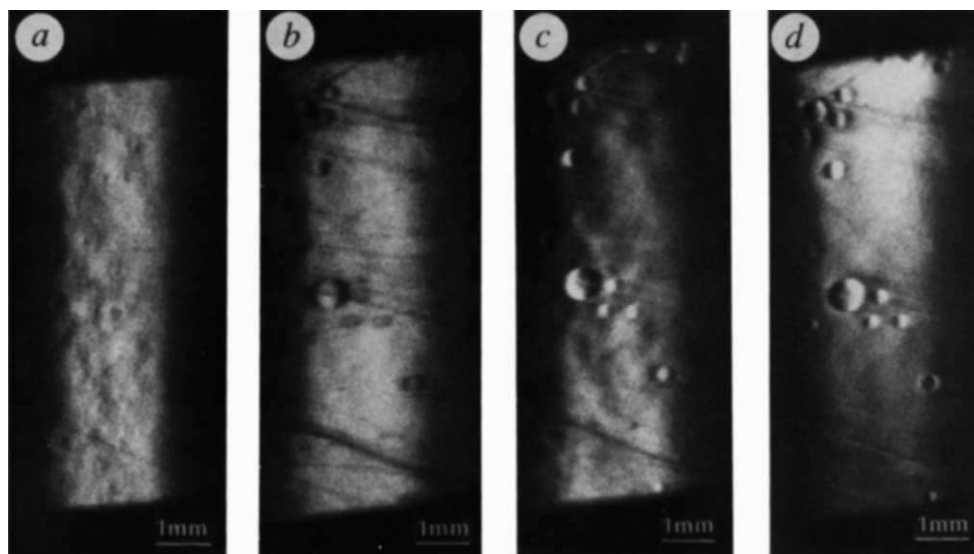
As a further demonstration of the technique, a test sample was constructed from glass fibres embedded in an acetone-based glue. The glass fibres yield little contrast in the conventional radiograph (Fig. 4a). Yet with phase-contrast imaging the fibres are clearly discernible (Fig. 4b). Furthermore, small air bubbles trapped in the glue during mixing are imaged as well. The contrast in each bubble is determined by the refractive-index difference, the curvature of the bubble and the offset angle of the analyser (Fig. 4c, d). The bubbles act like miniature X-ray lenses, producing convergence of the incident plane-wave which is detected by the analyser. The focal lengths of the bubbles are typically of the order of 100 m. Typical exposure times for Figs 3 and 4 are of the order of a few minutes with a conventional fine-focus tube source. This is considerably longer than is required for a direct radiographic

exposure from the same source because both high temporal and spatial coherence of the incident beam are involved in the present method.

The spatial resolution of the present method is limited by the divergence of the X-ray beam and the diffraction process in the crystals (T.E.G., manuscript in preparation, and T.J.D., manuscript in preparation). With an appropriate choice of X-ray source size, beam-defining slits and appropriate positioning of the sample, the effect of the beam divergence on the spatial resolution is minimized. Unlike the reflection of light from the surface of a mirror, the diffraction of X-rays occurs within the volume of the crystal which convolves adjacent rays (refs 15–17 and T.J.D., manuscript in preparation) and blurs image features. The diffracting volume of the crystal is determined by the dynamical extinction length, which increases with X-ray energy. Using asymmetric Bragg diffraction, the best spatial resolution for silicon with 8.048-keV X-rays is $\sim 0.2 \mu\text{m}$.

The degree of refraction of X-rays depends on the X-ray energy, E , and the electron density in the sample¹³. For example, for 8.048-keV X-rays, the difference in the refractive indices for protein and water is typically 1×10^{-6} which represents a refraction angle of ~ 0.2 arcsec for a protein–water interface, which might be considered a crude approximation for the scattering due to soft-tissue features. Although high stability and extreme precision in the alignment of crystals is required to measure such

FIG. 4 An example of hard X-ray phase-contrast imaging applied to a materials-science system. A thin-film test sample was prepared by mixing glass fibres with an acetone-based glue on a glass slide. The slide was removed after the glue had dried. a, Conventional radiograph, showing little detail other than imperfections in the first crystal used to expand and collimate the incident X-ray beam. b–d, Phase contrast images. b, The glass fibres are clearly discernible. Also seen are air bubbles trapped in the glue during mixing. c, d, As the analyser angle is altered (c, -0.6 arcsec; d, $+0.6$ arcsec) the contrast in the bubbles changes, indicating a change in direction of the X-rays across the bubble surfaces.



deviations, it is close to the range of angular resolution achieved in the present experiments.

The present methods of phase-contrast imaging differ from the earlier interferometry based technique developed by Bonse and Hart^{8,18} in that it requires no reference beam. Other methods of phase-contrast imaging that do not involve crystal-diffraction optics are also possible, for example by using a source of high spatial coherence combined with arrays of apertures before the sample and an accurate position analysing system for the transmitted radiation¹⁹.

The technique of X-ray phase-contrast imaging is sensitive to density gradients in the object and does not rely on absorption. In this respect it has applications in imaging weakly absorbing materials, such as carbon-based materials and biological systems. This raises the possibility of new techniques for non-invasive clinical diagnostic imaging. It may also be noted that the sensitivity of absorption contrast methods decrease with increase in X-ray energy as E^{-3} , whereas that of phase-contrast methods decrease only as E^{-2} . The latter methods therefore become relatively more sensitive at higher energies, leading to the possibility that in the phase-contrast approach the scattering medium will absorb a lower dose of X-rays at high energies for similar information to that obtained with conventional radiography, minimizing sample damage. □

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Dimercaptan–polyaniline composite electrodes for lithium batteries with high energy density

N. Oyama*, T. Tatsuma*, T. Sato* & T. Sotomura†

* Department of Applied Chemistry, Faculty of Technology, Tokyo University of Agriculture and Technology, Naka-cho, Koganei, Tokyo 184, Japan

† Energy Research Laboratory, Matsushita Electric Industrial Co. Ltd, Moriguchi, Osaka 570, Japan

THE development of low-cost, solid-state rechargeable batteries is of considerable technological importance. A key requirement of such batteries is that the density of energy stored electrochemically in the electrodes is high. In this context, the use of organic materials has attracted interest; they combine high theoretical energy storage capability with low weight and good mechanical strength. Here we report the development of a rechargeable lithium battery with a composite organic cathode based on a mixture of a dimer-

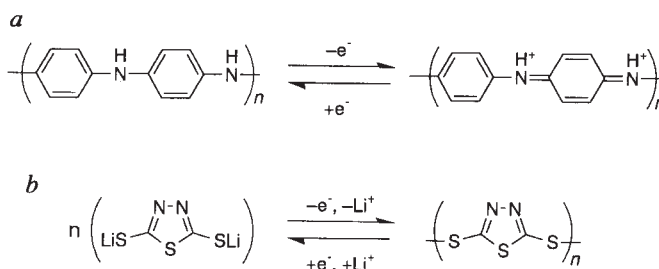


FIG. 1 Electrochemical reactions of PAN (a) and DMcT (b).

captan and polyaniline. The redox behaviour of the dimercaptan, which is normally too slow for practical applications^{1,2}, is accelerated when coupled to that of the polyaniline^{3–5} (which itself functions as an active cathode material). Intimate mixing of the two materials is achieved by casting them jointly from solution. The resulting electrode can be repeatedly charged to near its theoretical limit and discharged. The gravimetric energy density of our materials exceeds that of the oxide electrodes in commercially available lithium-ion batteries⁶, a feature that is likely to prove advantageous in applications where weight, rather than volume, is a critical factor.

Employment of a dimercaptan such as 2,5-dimercapto-1,3,4-thiadiazole (DMcT) as a cathode active material for lithium rechargeable (secondary) batteries has been reported by Visco and co-workers^{1,2}. As thiols are oxidized to give disulphides, dimercaptans can be polymerized by oxidation and depolymerized by reduction; but dimercaptans typically show only slow redox reactions at most electrode materials at room temperature. We have found that redox reactions of DMcT are accelerated³ on electrodes coated with electropolymerized polyaniline (PAN) and have reported fabrication of rechargeable batteries by coupling a DMcT–PAN composite cathode to the lithium anode^{3–5}. Although the cathode showed a relatively high gravimetric energy density (250 W h per kg cathode), all of the incorporated DMcT molecules were not reactive because the cathode consisted of a mixture of DMcT powder and chemically polymerized PAN powder, thus preventing intimate, molecular-level mixing of DMcT and PAN. Here we describe preparation of a cathode from an *N*-methyl-2-pyrrolidone (NMP) solution of DMcT and chemically polymerized PAN. The concentrated (DMcT + PAN)/NMP solution has a high viscosity like ink, so that a thin-film cathode can be prepared by using printing or painting techniques. As will be shown below, this new approach to cathode fabrication allows excellent molecular-level mixing of DMcT and PAN, leading to extremely efficient catalysis of the DMcT redox process by the PAN.

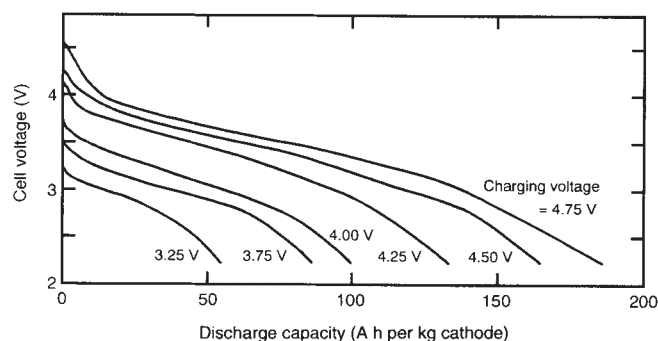


FIG. 2 Discharge curves for the lithium battery with the DMcT–PAN composite cathode (at 20 °C). The battery was charged at 3.25, 3.75, 4.00, 4.25, 4.50 or 4.75 V, and discharged at 0.1 mA cm⁻².

DMcT (2 g) was dissolved in NMP (5 g), and then chemically polymerized⁷ PAN (partially oxidized form (35%), Nitto Denko, Japan) (1 g) was dissolved in the yellow and viscous DMcT/NMP solution. The resulting dark and viscous 'ink' was spread on a carbon film and dried under vacuum at 80 °C for 1 h; the thickness and density of the resulting film was $\sim 20 \mu\text{m}$ and 1.5 g cm^{-3} , respectively. The film weight showed that $\sim 30\%$ of NMP was retained in the film, even after the evaporation. Solid polymer electrolyte (7–600 μm thick, 1.0 g cm^{-3}) was prepared as follows. A solution consisting of ethylene carbonate (7.9 g), propylene carbonate (10.5 g), acrylonitrile methylacrylate copolymer (mol ratio 20 : 1, 3.0 g) and LiBF_4 (2.3 g) was diluted with acetonitrile (3 g) and cast on a glass plate. The resulting film was dried under vacuum at 60 °C to remove acetonitrile, and then cooled to -20°C . Some properties of this solid electrolyte have been reported elsewhere⁵. The polymer-electrolyte film was sandwiched with the composite-cathode film and a lithium foil (300 μm thick) as an anode, and then sandwiched with current collectors (Ti foil) to give a test cell.

This fabricated battery was charged at a constant voltage (3.25–4.75 V) until the current became $<0.01 \text{ mA cm}^{-2}$. The discharge was performed at 0.1 mA cm^{-2} and cut off at 2.25 V (Fig. 2). Both charging and discharging were performed at 20 °C. Figure 2 shows that the discharge capacity increased with charging voltage monotonically from 3.25 to 4.75 V. A capacity of 185 A h per kg cathode was obtained at a charging voltage of 4.75 V. The theoretical maximum discharge capacity of PAN in the composite cathode is 65 A h per kg cathode (one electron is assumed to be consumed per aniline monomer unit, Fig. 1a). That of DMcT in the cathode is 159 A h per kg cathode (a two-electron reaction is assumed, Fig. 1b). The experimentally evaluated discharge capacity of 185 A h per kg cathode is thus $>80\%$ of the overall theoretical capacity of 224 A h per kg cathode. This confirms that not only PAN but also DMcT functions as a cathode active material in the present system.

When the charging voltage was 4.75 V, the average discharge voltage was $\sim 3.4 \text{ V}$, so that the gravimetric energy density of the composite cathode was $>600 \text{ W h per kg cathode}$. In a secondary battery equivalent to the most sophisticated National/Panasonic lithium-ion battery (commercially available), a LiCoO_2 cathode exhibits $\sim 400 \text{ W h per kg cathode}$ ⁶. That is, the energy density of the DMcT–PAN cathode is more than a factor of 1.5 better than that obtained previously using traditional approaches. Removal of the NMP left in the cathode would improve the

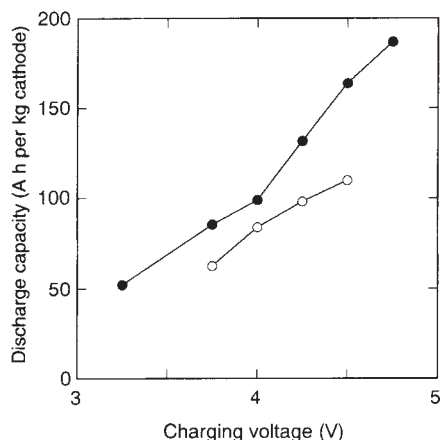


FIG. 3 Cyclic voltammograms of the DMcT–PAN film (2:1 by weight, solid line) and PAN film (dashed line)-coated gold electrodes (0.25 cm^2) obtained in 0.1 M LiClO_4 /propylene carbonate at 50 mV s^{-1} . The amount of PAN in the films is $3.2 \mu\text{g cm}^{-2}$ ($1/220$ of that for the charge-discharge experiment). The potential of Ag/Ag^+ is 3.4 V positive of Li/Li^+ .

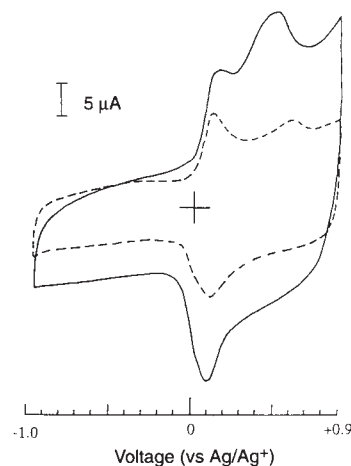


FIG. 4 Correlations between discharge capacity and charging voltage for the lithium battery with the new DMcT–PAN cathode (●) and the conventional mixed-powder cathode (○). Charging was cut off at 0.01 mA cm^{-2} ; discharging was performed at 0.1 mA cm^{-2} and cut off at 2.25 V.

energy density up to 900 W h per kg cathode. It should be noted, however, that this high gravimetric energy density will to some extent be offset by the low physical density of the organic materials. Our composite cathodes are therefore likely to prove useful in applications where weight, rather than volume, is critical.

To clarify the difference in electrochemical behaviour between DMcT in the composite cathode and dissolved DMcT on a gold or carbon electrode, cyclic voltammetry was performed. At a scan rate of 50 mV s^{-1} , DMcT showed a peak separation of $>1.0 \text{ V}$ owing to the slow electrode reaction at gold and carbon electrodes^{3,4} (data not shown). In contrast, the DMcT–PAN composite film coated on a gold electrode did not show such a peak separation but rather a simple enhancement of redox currents at the PAN potential (Fig. 3; the amount of DMcT and PAN cast on the electrode surface per unit area is $1/220$ of that for the charge-discharge experiments). This suggests that PAN mediates charge transfer between the carbon electrode and the DMcT moieties within the film, as we have described for the electropolymerized PAN–dissolved DMcT system³. The redox currents were almost proportional to the scan rate up to 100 mV s^{-1} . The single plateau seen in the discharge curve supports the above suggestion that the charge is transferred from the electrode to DMcT via PAN. Some chemical interaction between DMcT and PAN may be responsible for the charge-transfer reaction. Indeed, mixing DMcT with PAN gave some new Raman peaks. At present, we propose a chemical interaction between the thiolate of DMcT and the imine form of PAN, or an interaction between the π -orbitals of DMcT and PAN.

Figure 4 shows the correlations between discharge capacity and charging voltage for both the present DMcT–PAN cathode (data from Fig. 2) and a cathode prepared from a mixture of DMcT powder, PAN powder and the solid polymer electrolyte (mixed-powder cathode)^{4,5}. The two sets of data were obtained with identical test cells under the conditions described above. The average discharge voltages of two cathodes were almost the same. However, the discharge capacity of the new DMcT–PAN cathode was larger than that of the mixed-powder cathode. The difference was larger at a larger charging voltage. Furthermore, the mixed-powder cathode charged at 4.75 V could not be discharged. Above 4.0 V (versus Li/Li^+), PAN readily loses electrochemical activity and is difficult to reduce. We have found that the presence of DMcT in a PAN film dramatically enhances the maintenance of electroactivity of PAN. As to the new DMcT–PAN film cathode, molecular-level mixing of DMcT and PAN

results in almost all the PAN remaining electroactive, and the transfer of charge from almost all of the DMcT to PAN. This contrasts with the behaviour of the mixed-powder cathode, in which large domains of PAN become inactivated and a large proportion of the DMcT cannot undergo the redox reactions above 4.0 V because of poor molecular-level mixing of DMcT and PAN.

Because of this effect of maintaining the PAN activity, DMcT elongates the cycle life of the composite cathode. No deterioration in capacity was observed up to 30 cycles for the test cell with the new DMcT-PAN cathode charged at 4.50 V and discharged at 0.1 mA cm⁻² down to 2.25 V. Although the lithium anode gradually loses its activity at >30 cycles under the present conditions, the test cell retains 80–90% of its initial capacity even after 100 cycles when the inactivation of the lithium anode is unexpectedly small. However, the use of lithium-intercalated graphite or other anodes is beyond the scope of the present work. When the mixed-powder cathode was used, at least 15% of the capacity was lost after 30 cycles, even if the battery was charged only at 4.05 V and the discharge was cut off at 2.50 V. Therefore, the cycle performance of the new DMcT-PAN cathode is much better than that of the mixed-powder cathode; in addition to serving as the active cathode material, DMcT also plays a role in maintaining the activity of the composite cathode.

At present, the maximum useful current density of this composite cathode is ~0.1 mA cm⁻². Although this is an undesirably

small value, the present battery is a film type, so that a large electrode area can easily be obtained without unduly increasing the overall mass of the battery. We suspect that the low current density that can be achieved is due to the decreased conductivity of PAN at potentials above about 3.8 V versus Li/Li⁺. Thus we are currently examining the effect of adding another conducting polymer (such as a polypyrrole derivative, which also functions as a cathode active material) to the DMcT-PAN composite cathode to increase its conductivity. So far, this attempt has been successful in that the film conductivity has been significantly increased. Further molecular design of both mercaptans and conducting polymers is also underway, with a view to giving cathodes with better performance. □

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Seismic imaging of hotspot-related crustal underplating beneath the Marquesas Islands

David W. Caress^{*†}, Marcia K. McNutt[‡],
Robert S. Detrick[§] & John C. Mutter^{*}

^{*} Lamont-Doherty Earth Observatory of Columbia University, Palisades, New York 10964, USA

[†] SeaBeam Instruments, East Walpole, Massachusetts 02032, USA

[‡] Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

[§] Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543, USA

VOLCANISM in active continental rift zones^{1,2} or on rifted continental margins³ is frequently associated with unusually high lower-crustal seismic velocities, indicating the presence of large igneous intrusions at the base of the crust. The only comprehensive investigation of crustal underplating at an oceanic hotspot, beneath Hawaii^{4–6}, has yielded controversial results⁷. Here we report the results of seismic refraction experiments across the Marquesas Islands hotspot trace, which show that the island chain is underlain by crust 15–17 km thick, and that a large lower-crustal region (275 km wide and 2–8 km thick) has seismic velocities (7.3–7.75 km s⁻¹) indicative of crustal underplating. Wide-angle reflections occur near the base of the normal lower-crustal velocities and at the base of the underplating complex; we interpret these reflectors as the relict pre-hotspot Moho and the current post-hotspot Moho, respectively. The high-velocity material may be purely intrusive or may consist of a mixture of intrusive and pre-existing rocks. The volume of the high-velocity body is impressive, amounting to nearly twice the volume of volcanics erupted at the surface.

The Marquesas Islands have formed over the past 6 Myr as the Pacific plate moved northeastwards over a mantle

hotspot^{8–11}. The islands consist of isolated volcanic edifices rising from a 125-km-wide and 400-km-long archipelagic apron platform elevated 1,000–2,000 m above the surrounding sea floor^{12–14}. Figure 1 shows the location of line 1162, a seismic refraction and reflection profile across the Marquesas Islands. The refraction data consists of 22 unreversed sonobuoy profiles and 6 reversed profiles recorded by ocean-bottom seismometers (OBSs); all refraction data were obtained using a tuned 8,400 inch³ array of 20 airguns as a sound source.

All the OBS record sections (for example, Fig. 2a) show clear crustal (P) arrivals; Moho reflection (PmP) arrivals were observed on record sections from the easternmost four instru-

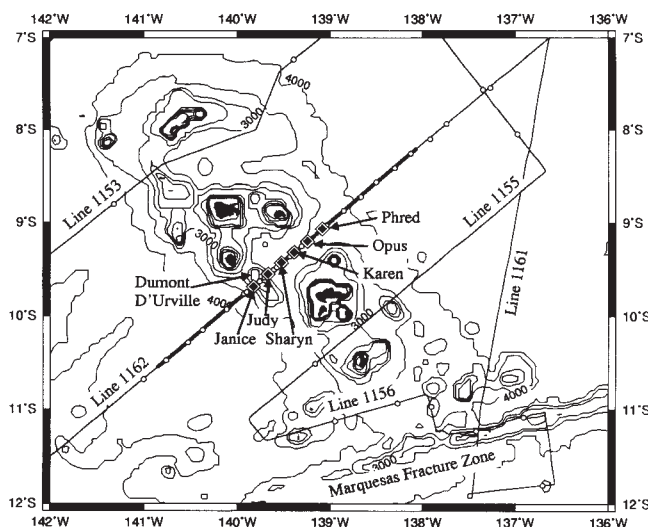


FIG. 1 Bathymetry of the Marquesas Islands¹⁴ and locations of seismic refraction profiles and seismic refraction experiments during cruises EW9103 and EW9204 on the RV *Maurice Ewing*. Sonobuoy stations are indicated by circles; OBS locations are indicated by diamonds.

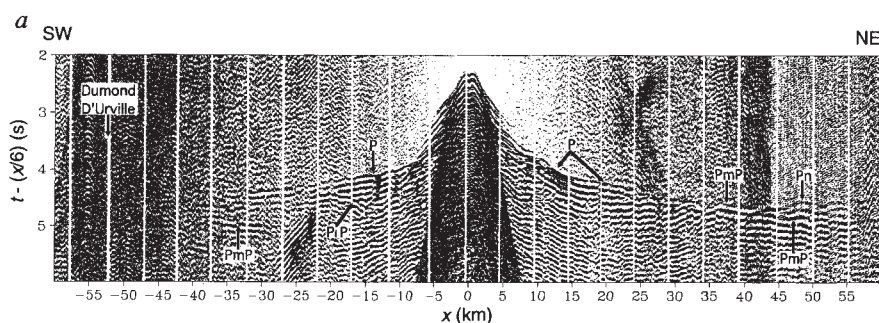
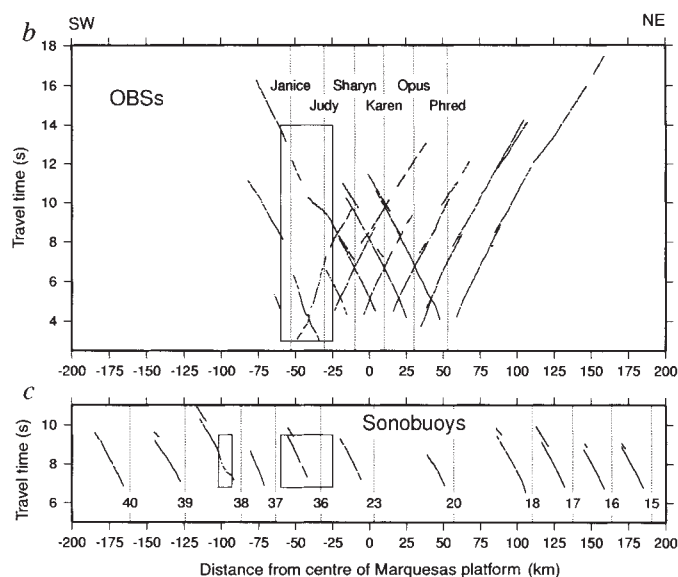


FIG. 2 *a*, Seismic refraction record section from the vertical channel of ocean-bottom seismometer (OBS) Opus, located 30 km northeast of the centre of the Marquesas platform. Travel time t in seconds is reduced by 6 km s^{-1} ; x is the shot distance from the OBS in km (northeast positive, southwest negative). In addition to the crustal refraction (P) and Moho reflection (PmP) phases, a mid-crustal wide-angle reflection (PiP) is observed on the southwestern side, and a mantle refraction phase (Pn) is observed to the northeast. *b*, Travel-time data obtained from OBS record sections, plotted as travel time versus the shot location (as distance from the centre of the Marquesas platform along line 1162; northeast positive, southwest negative). *c*, Travel-time data from sonobuoy record sections.



ments. A mid-crustal wide-angle reflection arrival (PiP) was also observed on record sections from the easternmost three instruments, but only beneath the eastern half of the platform. Water reverberation noise obscured the PmP and PiP arrivals on the western two instruments, limiting the resolution of the crustal structure and thickness beneath the western half of the platform. The crustal (P) refracted arrival is clearly visible in all the sonobuoy record sections. The pre-critical Moho reflection (PmP) can also be observed in sonobuoy record sections which were shot over oceanic crust with a 'normal' thickness of 5–6 km. With one exception (sonobuoy 36), the pre-critical PmP phase is not observed in sonobuoy data shot in crust which is thicker than 6 km (as on the Marquesas platform). The multichannel seismic reflection data collected along line 1162 imaged the internal structure and base of the archipelagic apron, but did not image Moho across the Marquesas platform.

The OBS record sections have yielded 7,617 travel-time picks, including picks from the P, PiP and PmP branches (Fig. 2*b*). In addition to the OBS data, we have used 5,459 P and pre-critical PmP travel-time picks from 11 sonobuoy refraction profiles (Fig. 2*c*) to model the crustal structure of a cross-section of the Marquesas hotspot trace.

The travel-time data have been analysed by forward ray-trace and tomographic inverse modelling using an interactive ray-tracing program which implements a two-dimensional ray-tracing and tomography scheme¹⁵. The sea floor and Moho are parametrized as first-order discontinuities; sea-floor depths are derived from multibeam echosounder data. Ray paths (Fig. 3*a*) and travel-time fits (Fig. 3*b*) are presented for the P arrivals observed at OBS Opus. Figure 3*c* shows the P ray-path distribution for the entire travel-time dataset, showing the scope of the crustal refraction ray-path coverage in the two-dimensional model. The observed P travel times and fitted to better than 0.04 s, except for ray paths complicated by the rugged bathymetry of Dumond

d'Urville and a small seamount ~50 km to the west of the platform's centre. Overall, 5,142 arrivals are fitted to an r.m.s. misfit of 0.037 s. The depths to the mid-crustal reflector and Moho were determined from the PiP and PmP reflection travel times (Fig. 3*d–g*), respectively, by ray-tracing through the crustal velocity structure determined from the P arrivals.

The velocity model given in Fig. 4 represents a cross-section of the Marquesas hotspot trace. The depths of basement (the top of the pre-existing oceanic crust as observed in seismic reflection data), the mid-crustal reflector, and Moho are overlaid on the velocity contours. Away from the platform, the crustal structure is consistent with a normal 6-km-thick oceanic crust, including lower-crustal velocities of $6.5\text{--}7.2 \text{ km s}^{-1}$. As the platform is approached, the pre-existing oceanic crust is buried by an increasing thickness of archipelagic apron, culminating in ~2 km of apron in the centre of the platform. Beneath the platform, travel times from multiple reversed refraction profiles require unusually high lower-crustal velocities of $\sim 7.5 \text{ km s}^{-1}$ at sub-basement depths of 8 km; turning P rays from a single unreversed profile penetrate to 11 km depth and suggest that velocities continue to increase. We conclude from our modelling that lower-crustal velocities of at least 7.75 km s^{-1} are required by the data; the best fit is obtained when the velocity increases to 7.9 km s^{-1} but this value depends somewhat on the model parameterization. The velocity is not directly constrained in the deepest crust beneath the platform; considering either substantially higher velocities or a low-velocity zone unlikely, we extend our best-fitting velocity model downward in the simplest fashion by assuming a uniform velocity of 7.9 km s^{-1} beneath the deepest-turning rays. The mid-crustal reflector occurs ~6–7 km beneath basement, roughly coincident with the increase from normal lower-crustal velocities ($\leq 7.2 \text{ km s}^{-1}$) to unusually high lower-crustal velocities ($\sim 7.5 \text{ km s}^{-1}$). Outside the platform Moho dips gently toward the islands, but beneath the platform

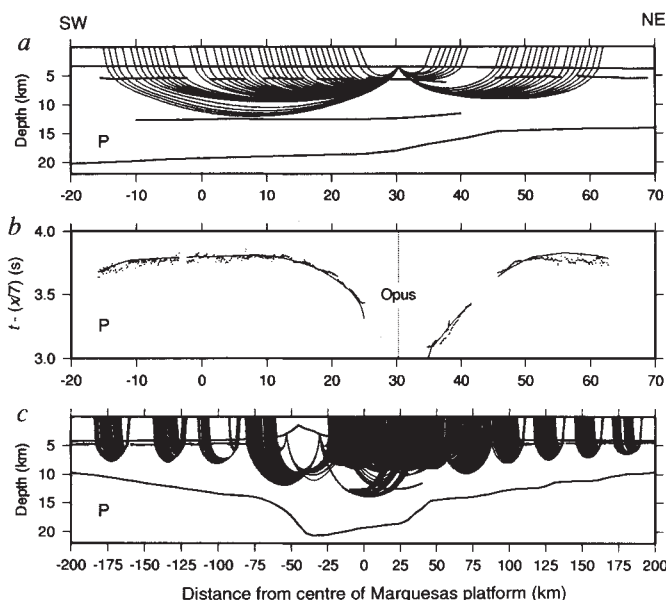
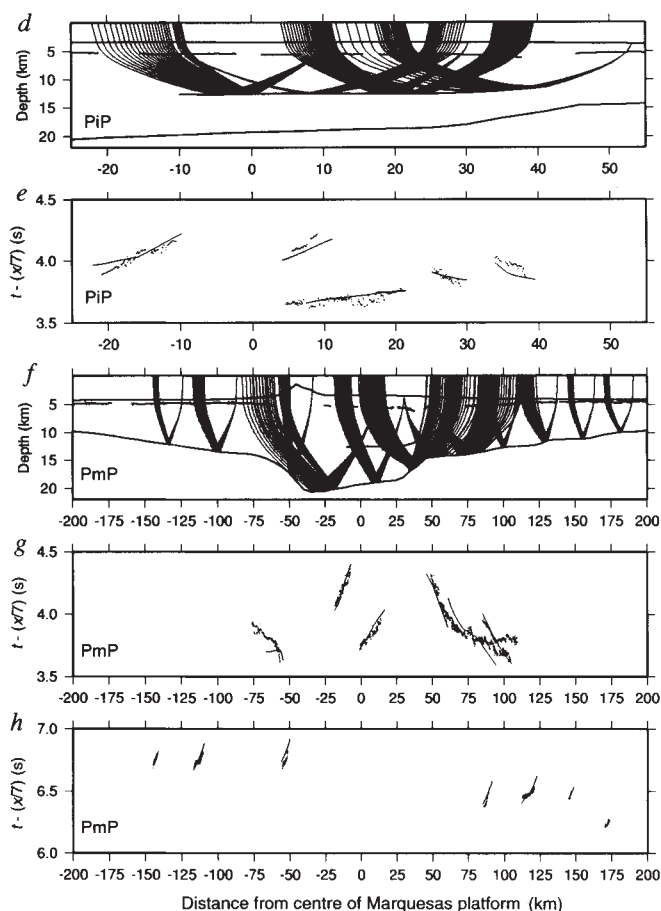


FIG. 3 Examples of ray paths and travel times used in modelling. The locations of the sea floor, basement, the mid-crustal reflector and Moho (bold lines) are overlain on the ray-path plots. Travel times are reduced by 7 km s^{-1} and located according to distance of shot from the centre of the Marquesas platform. Observed travel times are shown as dots, modelled results as lines. *a*, Ray paths used to model P arrivals observed on OBS Opus. *b*, Results of modelling P travel times from OBS Opus. *c*, P ray paths from all OBSs and sonobuoys used in modelling. *d*, Ray paths used to model the PiP arrivals. *e*, Results of modelling PiP arrivals. *f*, Ray paths used to model the PmP arrivals. *g*, Results of modelling PmP arrivals observed on OBSs. *h*, Resulting of modelling PmP arrivals observed on sonobuoys.



Moho is depressed an additional 4–6 km; the total crustal thickness beneath the platform is 15–17 km. Pn arrivals observed on two OBS record sections constrain the upper-mantle velocity east of the platform to be at least 8.1 km s^{-1} , and the observation of large PmP arrival amplitudes indicates that Moho is well defined beneath the platform.

We interpret the high-velocity lower-crustal body as evidence for large-scale crustal underplating that took place during the hotspot volcanism in the Marquesas Islands. This high-velocity root is not coincident with any particular volcanic edifice, but rather extends under the entire Marquesas platform, with lesser anomalies extending at least 75 km out beneath the flanks. Because the mid-crustal reflector occurs at the base of a velocity sequence equivalent to that of normal oceanic crust, we tentatively identify the reflector as the relict pre-hotspot Moho. Analysis of gravity and basement deflection profiles coincident with lines 1153, 1162 and 1155 (Fig. 1) across the Marquesas Islands provide independent evidence for a large, unusually dense lower-crustal body beneath the length and the width of the Marquesas platform¹⁶. Assuming that the dimensions of the underplated body are similar along the length of the island chain, the volume of underplated material is $620,000 \text{ km}^3$. Analysis of the bathymetry and seismic reflection data in the Marquesas⁹ yields volume estimates of $90,000 \text{ km}^3$ for the existing volcanoes and $240,000 \text{ km}^3$ for the material shed from the islands. Thus the volume of the underplated body in the Marquesas is 1.9 times the total volume of material erupted at the surface.

The seismic velocities in the underplated body are intermediate between the velocities attributed to gabbros ($6.7\text{--}7.2 \text{ km s}^{-1}$) and peridotites ($7.8\text{--}8.3 \text{ km s}^{-1}$)¹⁷; these results indicate either a mixture of mafic and ultramafic rocks at scales smaller than the resolution of the seismic refraction data (which is no better than 2 km in the underplated body), or rocks with a composition intermediate between mafic and ultramafic (for example, pic-

rites). Our preferred model is that the underplating results from massive intrusions associated with the hotspot volcanism; the extent to which the underplated material incorporates previously existing mantle or crustal rocks is unclear.

The similarity between the Marquesas crustal cross-section shown in Fig. 4 and the model for the Hawaiian islands proposed

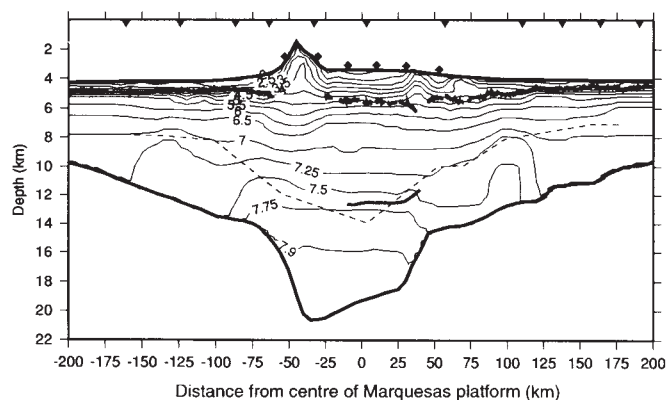


FIG. 4 Seismic velocity model for a cross-section of the Marquesas Islands. Sonobuoy stations are indicated by triangles, OBS locations by diamonds. The locations of the sea floor, the top of the pre-existing crust (basement), the relict Moho and the current Moho (bold lines) are overlain on the velocity contours (shown in km s^{-1}). The approximate location of the deepest penetration of turning P ray paths is shown as a dashed line; velocity values below this line are not directly constrained by travel time observations and are thus conjectural. Velocities in the model continue to increase just below the deepest P rays because turning rays imply a velocity gradient at the deepest penetration. The composition of the large high-velocity ($>7.2 \text{ km s}^{-1}$) lower-crustal body is unknown; the underplated material may be purely intrusive or may be a mixture of intrusive rocks with pre-existing mantle peridotites.

by Watts *et al.*⁴ is striking. Seismic refraction data from the Kerguelan Islands on the Kerguelan plateau resolved lower-crustal velocities of 7.2–7.5 km s⁻¹ at depths of 15–20 km which were interpreted as evidence for underplating, but did not constrain the location or nature of the crust–mantle boundary¹⁸. The recent discovery of mafic granulite xenoliths on Kerguelen¹⁹ derived from depths of 20–45 km indicates that this very large oceanic plateau may be underlain by a much thicker underplating sequence than the Marquesas. Thus the existing observations strongly suggest that underplating is an integral part of intra-plate volcanism in oceanic lithosphere. Underplating has also been inferred in rift settings¹³, suggesting that crustal underplating is a fundamental process associated with volcanism in general. □

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An Early Miocene anthropoid skull from the Chilean Andes

John J. Flynn*, André R. Wyss†, Reynaldo Charrier‡ & Carl C. Swisher§

* Department of Geology, Field Museum, Roosevelt Road at Lake Shore Drive, Chicago, Illinois 60605, USA

† Department of Geological Sciences, University of California, Santa Barbara, California 93106, USA

‡ Departamento de Geología, Facultad de Ciencias Físicas y Matemáticas, Universidad de Chile, Casilla 13518, Correo 21, Santiago, Chile

§ Berkeley Geochronology Center, 2455 Ridge Road, Berkeley, California 94709, USA

PARTLY because of their poor fossil record, the relationships of neotropical platyrrhine monkeys to other groups of primates and to each other remain perhaps the most poorly known for any major primate clade¹. Here we report the discovery of a complete platyrrhine skull from the Andes of central Chile, by far the best preserved Tertiary primate cranium from South America. This find, coupled with recent phylogenetic analyses of higher groups of anthropoid primates^{2–4}, has the potential to revise substantially our understanding of platyrrhine interrelationships, indicating, among other points, significant modification to reconstruction of the ancestral platyrrhine morphotype and a likely African origin for New World monkeys. A ⁴⁰Ar/³⁹Ar radioisotopic date directly associated with the skull indicates an Early Miocene age⁵, marking the first report of South American mammals of this age from outside Argentine Patagonia. Finally, this discovery demonstrates

the enormous potential of vastly distributed, but virtually untapped, Andean volcanoclastic deposits to yield further insights into the origin and diversification of South American primates.

Fossil mammals from the Chilean Andes have come to light only recently. Our previous work⁶ in high-elevation volcanoclastic deposits of the Abanico (Coya-Machali) Formation near Termas del Flaco (35° S) produced two Palaeogene fossil mammal faunas, the first significant palaeontological discoveries for this major central Andean rock unit. The Abanico Formation strikes north–south parallel to the Andean Main Range and is broadly exposed over several hundred kilometres. Work north of Termas del Flaco during January 1994 yielded two younger mammal faunas, including recovery of an extraordinary platyrrhine skull. Collectively, these four Andean faunas demonstrate the geographically, stratigraphically and temporally widespread occurrence of often exquisitely preserved Cenozoic mammals in the Abanico Formation, which is remarkable considering that this unit had long been regarded as barren of age-diagnostic fossils and as probably Late Cretaceous in age⁶.

Primates Linnaeus 1758

Anthropoidea Mivart 1864

Platyrrhini E. Geoffroy, 1812

Incertae sedis

Chilecebus carrascoensis gen. et sp. nov.

Holotype. SGOPV 3213 (Museo Nacional de Historia Natural, Vertebrate Palaeontology collections, Santiago, Chile). A virtually complete, little deformed skull with complete upper dentition missing only the buccal edges of left C-M2 (Fig. 1). The dentition was prepared mechanically; as the specimen is preserved in an extremely hard volcanoclastic matrix, other anatomical features were elucidated using computed axial tomography (Fig. 1).

Etymology. Chile, referring to its country of origin, cebus, a common suffix for platyrrhine primates; honoring Gabriel Carrasco, an extraordinary fossil collector and field party member who discovered the specimen.

Stratigraphic horizon and locality. The locality occurs in tightly folded levels of the Abanico Formation, along the Río Las Leñas (±100 km south-southeast of Santiago, 60 km north-northeast of Termas del Flaco). Well preserved mammalian fossils, including skulls of notoungulates and other eutherians, are abundant.

Age. The volcanic fossiliferous horizon permits direct radioisotopic dating of the primate, as the dated sample and the skull are derived from the same ~400 cm³ matrix block. An ⁴⁰Ar/³⁹Ar analysis indicates a 20.09 ± 0.27 Myr age for the new platyrrhine (Table 1). Preliminary biostratigraphic evidence (including abundant rodents and hypsodont notoungulates) also indicates that this fauna is younger than both previously reported⁶ Palaeogene faunas in the Termas del Flaco area, and is consistent with an Early Miocene (Late Deseadan through Colhuehuapian SALMA) age assignment⁵.

Diagnosis. Small (~1,000 g (average of best-fit estimates of body size from three skull measures)⁷ to 1,200 g (average of best-fit estimates from areas of nine upper teeth, ranging from 751–1,871 g)⁸) platyrrhine with 2133 dental formula, moderate-sized orbits (~9 mm), bony postorbital septum, cranial capacity in the range ~6.6 cm³ (braincase linear dimension estimators)⁷ to 7.5 cm³ (graphic double integration of braincase volume obtained from CT scan)⁹, and short skull relative to estimated body size⁷. Dental measurements (mesio-distal/bucco-lingual in mm) are: I1, >1.8/2.92; I2, 2.12/2.64; C, 2.58/2.78; P2, 1.75/3.50; P3, 1.96/4.57; P4, 2.20/4.85; M1, 2.89/4.95; M2, 3.00/4.55; M3, 2.41/3.87 (Fig. 1). *Chilecebus* is distinguished from all Recent and fossil platyrrhines in the following combination of characters: skull low-vaulted in profile, lacking sagittal crest; upper dentition defines broad, slightly 'V'-shaped arcade (palatal width increasing posteriorly between diverging tooth rows); incisors subequal in size, spatulate and markedly procumbent; canine very small, separated from I2 by a short ~1 mm diastema;

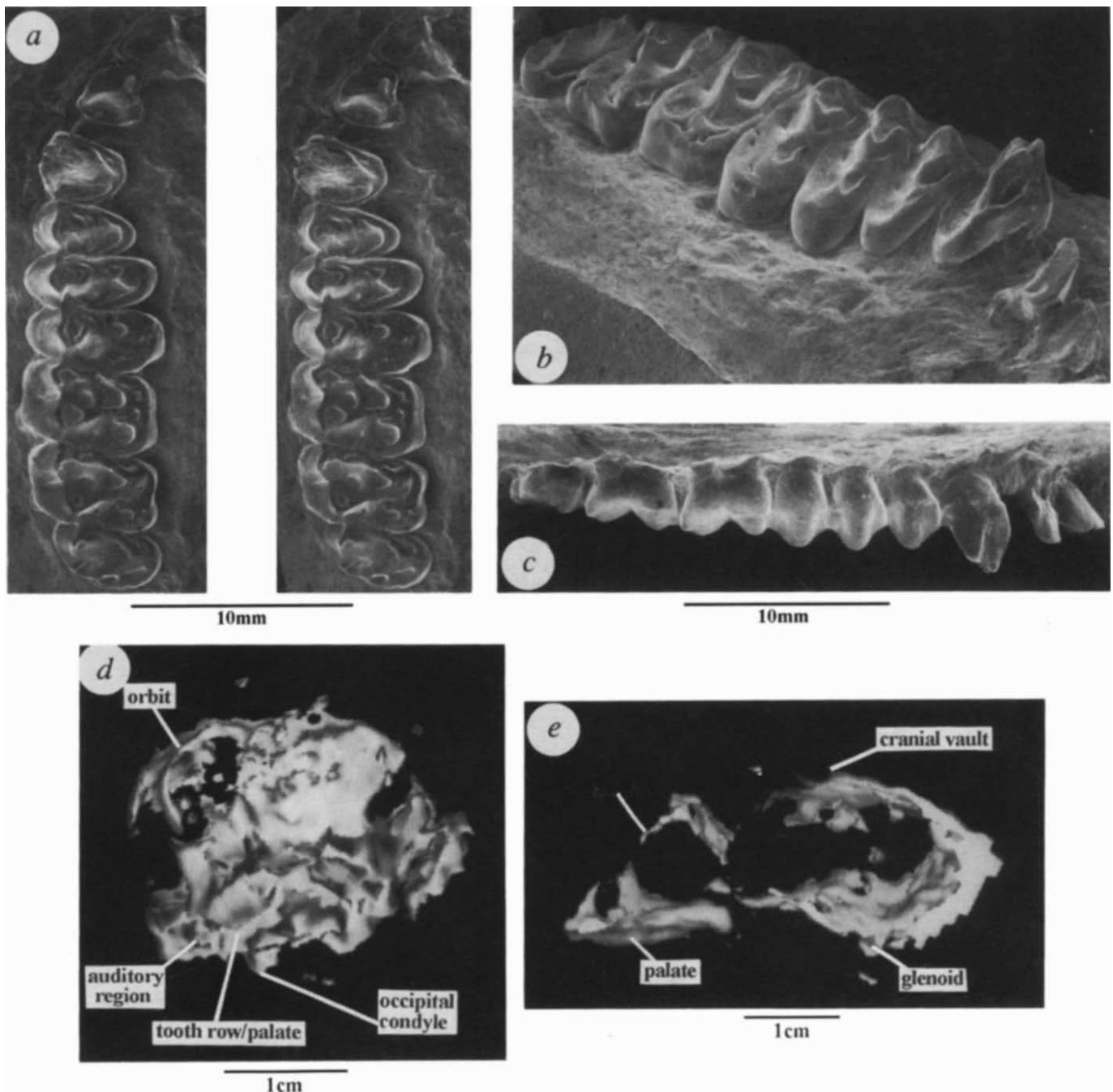


FIG. 1. Holotype (SGOPV 3213) of *Chilecebus carrascoensis* gen. et sp. nov. a, Stereopair, crown; b, buccal; c, oblique SEM views of right upper dentition; and computed tomography scan, three-dimensional images, anterior view d, and internal lateral view, from midline outwards e. All but the dentition remains encased in extremely hard volcanoclastic matrix. Apparent discontinuity of the skull in CT images is due to the resolving power of the imaging instrument; symmetry of landmarks on the scans and visible parts of the specimen indicate it is little deformed. Subequal spatulate incisors are notably procumbent, approaching the condition seen in pitheciines, and probably characterizing *Soriacebus*. The canine is small for a platyrrhine, even accounting for possible sexual dimorphism; like the incisors, the canines bear a strong posterior cingulum. A short ~1 mm diastema separates I2-C. P2 is single or double rooted, unicusuate. A faint swelling at the anterolingual base of the paracone might indicate a paraconule. P3, 4 are markedly larger than

P2; both bear a large paracone, subequal pre- and post-paracristae and small protocone. P4 is a more robust tooth, distinguished chiefly by strong posterolingual cingulum and greater anteroposterior width. M1, 2 are quadrate, possessing well developed hypocones and weak prepara- and postproto-cristae; metaconules are lacking. The metacone is larger than the paracone on M1; the reverse is true on M2. The mesial fovea is larger on M2. M1 lacks a parastyle due to the paracone's anterior position. M1, 2 bear a short postmetacrista with a small mesostylar cuspsule. M3 is much smaller and more ovoid than preceding molars; from the large anteriorly situated paracone there is a steeply sloping postparacrista bearing a small posterobasal cusp—an incipient/ vestigial metacone or metastyle, but not a typical metacone; the postprotocrista sweeps to the posterior tooth edge, extending to the base of this small cusp and forming a broad posterobuccally deepening trigon basin.

P2 one or two rooted, unicusuate, strong postero-lingual talon basin, narrower transversely than succeeding premolars; P3 rectangular in occlusal outline, similar to P4 in bearing small protocone but lacking hypocone; M1-2 quadrate, with well

developed hypocones, weak prepara- and postproto-cristae, lacking metaconule; M3 ovoid, lacking both typical hypocone and metacone, with strong lingual cingulum and posterolingual shelf (Figs 1, 2). *Chilecebus* is easily distinguished from other

TABLE 1 $^{40}\text{Ar}/^{39}\text{Ar}$ data for the Monkey Plagioclase

L	$^{40}\text{Ar}^\dagger$	Ca/K ‡	$^{40}\text{Ar}/^{39}\text{Ar}^\S$	$^{36}\text{Ar}/^{39}\text{Ar}$	$^{40}\text{Ar}^*/^{39}\text{Ar}$	% $^{40}\text{Ar}^*$	Age (Myr)	s.d. (1σ) $ $
8065-01	4.98	10.75	3.69	0.0074	3.32	88.8	20.02 $\pm$ 0.75	
8065-02	3.66	11.73	4.36	0.0103	3.32	75.1	20.05 $\pm$ 0.72	
8065-03	3.07	11.69	4.24	0.0098	3.34	77.6	20.14 $\pm$ 0.87	
8065-05	3.45	8.90	3.91	0.0071	3.32	83.8	20.01 $\pm$ 0.59	
8065-06	4.08	12.03	5.83	0.0154	3.32	56.0	20.02 $\pm$ 0.72	
8065-07	3.69	16.72	5.94	0.0184	3.35	55.2	20.23 $\pm$ 1.40	
8065-09	6.73	11.91	4.58	0.0110	3.35	72.1	20.22 $\pm$ 0.54	
					Arithmetic mean		20.10 $\pm$ 0.10	
					Weighted mean		20.09 $\pm$ 0.27 $ $	
Analysis not used								
8065-04	6.14	14.52	8.13	0.0234	3.68	44.4	22.20 $\pm$ 1.05	

The age of the Chilean platyrrhine was determined by $^{40}\text{Ar}/^{39}\text{Ar}$ laser total fusion of plagioclase separated from the fossil-containing block. Analyses follow¹⁹, except where noted below. The 'Monkey Plagioclase' and Fish Canyon Sanidine Monitor Mineral were enclosed in a thin cadmium liner and irradiated for 1.5 h in the Oregon TRIGA Reactor. J was determined from replicate single crystal analyses of co-irradiated monitor mineral using an age of 27.84 Myr, similar to that recommended in ref. 19, but slightly modified because of in-house (BGC) intercalibration with MMhb-I with a published²⁰ age of 520.4 $\pm$ 1.7 Myr. J for irradiation $117\text{B} = 3.36 \times 10^{-3} \pm 1.94 \times 10^{-6}$, $^{40}\text{Ar}/^{36}\text{Ar}$ mass discrimination = 1.005 ± 0.0002 . Ca and K corrections were determined from laboratory salts: ($^{36}\text{Ar}/^{37}\text{Ar}$)Ca = $2.742 \times 10^{-4} \pm 9.7 \times 10^{-6}$, ($^{39}\text{Ar}/^{37}\text{Ar}$)Ca = $6.852 \times 10^{-4} \pm 2.4 \times 10^{-5}$ and ($^{40}\text{Ar}/^{39}\text{Ar}$)K = $9.0 \times 10^{-2} \pm 3.0 \times 10^{-4}$. Decay constants were as recommended in ref. 21. Released gases purified by two SAES C-50 getters operated at $\sim 400^\circ\text{C}$. The new Las Leñas fauna represents the first fossils of this age from Chile and the Andean chain. Biostratigraphic evidence and the new $^{40}\text{Ar}/^{39}\text{Ar}$ date establish that the 'eastern strip' of the Abanico Formation, generally considered Late Cretaceous but poorly constrained, is substantially younger than once supposed⁶. These new 20.09 Myr deposits provide direct geochronological control on the late Oligocene–early Miocene (the younger limit of the Deseadan, the entire Colhuehuapian and its older and younger limits, and the older limit of the Santacrucian) portion of the SALMA sequence⁵. L, BGC laboratory analysis number.

† Moles $\times 10^{-15}$.

‡ Ca/K calculated from measured $^{37}\text{Ar}/^{39}\text{Ar}$ value using TRIGA irradiation production values of 0.86 for $^{39}\text{Ar}_\text{K}$ (mol $^{39}\text{Ar}/\text{g K} - \text{h} \times 10^{-10}$) and 0.42 for $^{37}\text{Ar}_\text{Ca}$ (mol $^{37}\text{Ar}/\text{g Ca} - \text{h} \times 10^{-10}$).

§ Blank corrected.

* Radiogenic ^{40}Ar .

$||$ 1σ , one standard deviation.

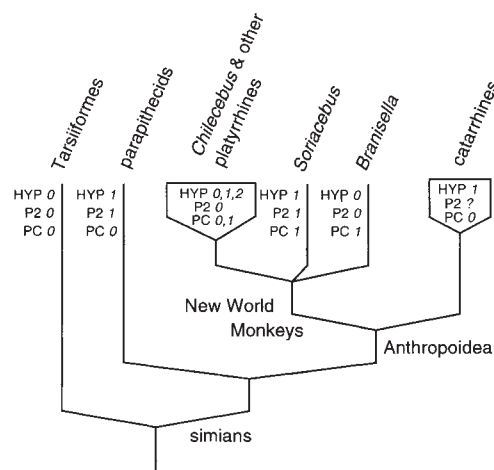
$||$ Replicate analyses weighted mean and standard error (s.e.) follows ref. 22.

early-middle Miocene platyrrhines (and all other platyrrhines), differing most notably from them in cranial and dental features listed above, and the following: from *Branisella*¹⁰ in more transverse P4, more quadrate molars, stronger M1-2 hypocones, lacking M3 metacone; from *Dolichocebus*¹¹ in weaker molar paraconules, lacking M3 metacone, smaller size; from *Tremacebus*¹² in lacking molar pericone, M1 hypocone present, lacking M3 metacone, smaller size; from *Carlocebus*¹³ in much smaller size, lacking P3-4 hypocones, smaller molar hypocones, absence of M3 metacone; from *Homunculus*^{14,15} in procumbent

incisors, shorter diastema, lacking M3 metacone, smaller size; from *Soriacebus*¹³ in smaller canine, lacking premolar hypocones, P2 not triple-rooted, smaller size.

In profile, the vaulting of the skull of *Chilecebus* (Fig. 1) is lower apparently than in modern platyrrhines, and the few fossils for which this condition may—with varying degrees of reliability—be assessed. As in other Miocene platyrrhines, but in contrast to some early fossil African simians, there is no sagittal crest (Fig. 1). The somewhat ovoid orbits are small (~ 9 mm, Fig. 1; best measured from axial CT views; not shown), but

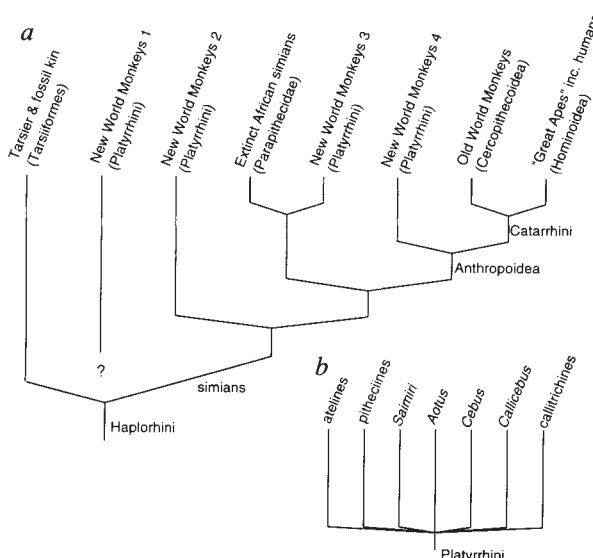
FIG. 2 Simplified cladogram of haplorhine relationships illustrating character transformations frequently employed in platyrrhine phylogenetics (modified from ref. 2). This exercise calls into question several features generally accepted as ancestral for platyrrhines. Beyond acceptance of a few subfamilial and suprageneric groupings, there is little consensus regarding higher level platyrrhine relationships. Taxa frequently cited as possible outgroups to crown group platyrrhines include *Branisella*¹⁷ and *Soriacebus*³. *Soriacebus* has been regarded as primitive with respect to other platyrrhines in possessing a triple-rooted P2 (P2 0), a condition argued to be ancestral for platyrrhines³. Outgroups to platyrrhines bear double- or single-rooted P2s (P2 1). Catarrhines cannot be evaluated for this feature because P2 is lacking, complicating character optimization. Triple-rooted P2s are conventionally considered ancestral for Anthropoidea (with reversal in platyrrhines exclusive of *Soriacebus*). Equally parsimoniously it may be argued that triple-rooted P2s are convergent in parathithecids and *Soriacebus*. Choice between these alternatives partly depends on phylogenetic placement (based on evidence independent of this character) of *Soriacebus*. Triple-rooted P2s are not necessarily ancestral for platyrrhines or primitive. There is disagreement concerning the ancestral hypocone condition and upper molar shape in platyrrhines. Three conditions are recognized: (Hyp 0) well developed hypocone and quadrate molars (most platyrrhines); (Hyp 1) small hypocone and moderately triangular molars (*Branisella* and the closely related or congeneric *Szalotavus*); (Hyp 2) lacking hypocone and triangular molars (callitrichines). Rosenberger and others²³ suggested that condition 1 might be ancestral for platyrrhines, based primarily on the antiquity of *Branisella* and retention of other assumed primitive dental features. Hershkovitz^{12,24} has long maintained that condition 2 is ancestral for platyrrhines. Condition 0



characterizes outgroups, suggesting this is ancestral for platyrrhines, and resemblances between conditions 1 and 2 are synapomorphic, contrary to prevailing opinion. Paraconules (PC 1) are stated to characterize platyrrhines ancestrally because they occur in *Dolichocebus* (uniquely, among platyrrhines)¹¹ and outgroups. Unless *Dolichocebus* represents a basal platyrrhine (something never proposed), lack of paraconule (PC 0) represents the most likely ancestral condition for platyrrhines. These examples highlight present uncertainty about the ancestral platyrrhine morphotype and character polarization.

BOX 1 Summary of competing hypotheses of inter-relationships among major groups of haplorhine (tarsiiform + anthropoid) primates (a) and among living platyrrhine (New World) monkeys (b)

Platyrrhine placement 1, most ardently advocated by ref. 24 (but not in explicit cladistic terms), implies the multiple independent (polyphyletic) origins of the simian 'higher-primate' taxa generally termed 'Anthropoidea', a view consistent with a separate 'prosimian' ancestry and North American source area for the Platyrrhini. We are not aware of shared derived characters supporting Platyrrhini 1, the acceptance of which dictates convergent evolution of several features (post-orbital closure for example) usually considered synapomorphic for simians. In placement 2, platyrrhines are regarded²⁵ as the sistergroup to remaining anthropoids, a phyletic position compatible with either a North American or African origin of the group. Derivation of platyrrhines from fossil African parapithecids (Platyrrhini 3) has also been advocated²⁶. The shortest length topology of a recent parsimony analysis² is shown as Platyrrhini 4. Information from the new platyrrhine, *Chilecebus*, the oldest well preserved primate skull from the continent, argues in favour of Platyrrhini 4, and thus an African source region for the New World monkeys. Our usage of the name 'Anthropoidea' merits brief comment. There is growing consensus²⁷ that well known taxonomic names (such as Anthropoidea) are best applied to crown clades. We propose a crown clade-based phylogenetic definition for Anthropoidea (in disagreement with ref. 28), the clade stemming from the most recent common ancestor of Platyrrhini and Catarrhini. We use 'Simiæ' or the vernacular 'simians' (typically used in contrast to a gradual grouping of 'more primitive prosimians'; see ref. 7) to refer to all primates more closely related to anthropoids than to *Tarsius*. Similarly we advocate crown clade definitions of the names Platyrrhini, Catarrhini and Primates. *b*, Recent morphologically based cladistic studies^{3,29,30} have aimed to resolve phylogenetic relationships among fossil and living platyrrhines; a strict consensus phylogeny of living forms derived from these three studies is shown. Besides monophyly of each of three 'subfamilial' groupings (Callitrichinae, Atelinae, Pitheciinae), there is no agreement about higher-



level phylogenetic relationships among the platyrrhines. Affinities of the few (and generally fragmentary) pre-Late Miocene fossil New World monkeys are even more poorly understood, and are omitted here for simplicity. Unresolved problems include identification of higher-level groupings among crown clade platyrrhines, and whether various early fossil New World monkeys (for example, *Branisella*, *Chilecebus*) are in fact members of the crown clade or represent outgroups. In representing by far the best preserved early New World monkey, additional analysis of *Chilecebus* promises to clarify the ancestral morphology of the group as a whole, which in turn may shed light on the poorly understood origin, timing and pattern of diversification of this palaeontologically under-represented group of anthropoid primates.

comparable to some early haplorhines. There clearly is an expanded osseous postorbital septum, but it is not yet possible to determine its degree of ventral closure. In the ear region *Chilecebus* resembles other platyrrhines (and the African fossil simians *Aegyptopithecus* and *Apidium*), as the ectotympanic is not expanded into a meatal tube.

The record of New World monkeys is exceptionally poor. Most fossil platyrrhines are known from isolated teeth and jaws; a handful are known from edentulous, distorted or otherwise badly preserved skulls or fragments. The exquisitely preserved *Chilecebus* represents not only the best known early South American primate, but the most complete skull for any Tertiary platyrrhine as well. *Chilecebus* appears to have been diurnal (orbital index⁷ <0.8), as are all living platyrrhines (except *Aotus*), proportionally smaller-brained (index of cranial capacity⁷ about 2.0) than all living haplorhines (but similar to fossil 'tarsioids' and the African anthropoid *Aegyptopithecus*⁷), and frugivorous or possibly folivorous (based on body-size, low-crowned teeth with bulbous cusps and incisor morphology¹⁶). *Chilecebus* is unusual in its small size, low skull vault, small canine (even considering possible sexual dimorphism, the uncommon degree of incisor procumbency and the peculiar mixture of primitive and derived cheek tooth features). Other recently described early platyrrhines display similar mosaic character combinations^{1,11}, leading to significant uncertainties regarding placement of these taxa within existing phylogenies. Difficulties stem from the scant fossil record of early platyrrhines in general and the poor resolution of relationships among even the living forms (Box 1). *Chilecebus* shows discordant derived resemblances to a variety of fossil and living platyrrhines; definitive higher-level placement awaits clarification of platyrrhine phylogeny. Even so, the new evidence from *Chilecebus* and a provisional understanding of platyrrhine phylogeny, are sufficient to call into question several dental features conventionally accepted as characterizing platyrrhines ancestrally, including

a triple-rooted P2, lack of molar hypocone and presence of paraconules (Fig. 2).

Chilecebus confirms the prediction^{1,17} that new pre-middle Miocene discoveries would reveal an early, diverse platyrrhine radiation. At 34° S, *Chilecebus* also extends the geographic (lower latitude and more westerly) and environmental (high-gradient, intra-montane Andean, with proximal volcanoclastic substrate and little paleosol formation) sampling of early-middle Miocene platyrrhines, previously known only from high latitudes (>42° S) of Argentine Patagonia. Discovery of *Chilecebus* marks, and tightly constrains the minimum age for, the earliest distribution of New World monkeys outside the tropics, as none are known from well sampled older higher latitude deposits (such as Argentine Deseado) and the Argentine Colhuehuapien platyrrhines currently are undated. This may mark the beginning of an early-middle Miocene climatic amelioration (warmer, less seasonal) and expansion of forest/riparian habitat¹⁸. Because of its broad age range (at least early Eocene-early Miocene), vast geographical extent, unique depositional environment, and exceptional fossil preservation, the Abanico Formation of the Central Chilean Andes offers an unexcelled opportunity to further basic understanding of the early diversification of platyrrhines. □

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Sex differences in the functional organization of the brain for language

Bennett A. Shaywitz*†, Sally E. Shaywitz*, Kenneth R. Pugh*†, R. Todd Constable§, Pawel Skudlarski§, Robert K. Fulbright§, Richard A. Bronen§, Jack M. Fletcher||, Donald P. Shankweiler†, Leonard Katz† & John C. Gore§¶

Departments of * Pediatrics and † Neurology, Yale University School of Medicine, PO Box 208064, New Haven, Connecticut 06510-8064, USA

‡ Haskins Laboratories, 270 Crown Street, New Haven, Connecticut 06511, USA

§ Department of Diagnostic Radiology, Yale University School of Medicine, PO Box 208042, New Haven, Connecticut 06520-8042, USA

|| Department of Pediatrics, University of Texas Medical School, 6431 Fannin, Houston, Texas 77030, USA

¶ Department of Applied Physics, Yale University, Becton Engineering and Applied Science Center, PO Box 208284, New Haven, Connecticut 06520-8284, USA

A MUCH debated question is whether sex differences exist in the functional organization of the brain for language^{1–4}. A long-held hypothesis posits that language functions are more likely to be highly lateralized in males and to be represented in both cerebral hemispheres in females^{5,6}, but attempts to demonstrate this have been inconclusive^{7–17}. Here we use echo-planar functional magnetic resonance imaging^{18–21} to study 38 right-handed subjects (19 males and 19 females) during orthographic (letter recognition), phonological (rhyme) and semantic (semantic category) tasks. During phonological tasks, brain activation in males is lateralized to the left inferior frontal gyrus regions; in females the pattern of activation is very different, engaging more diffuse neural systems that involve both the left and right inferior frontal gyrus. Our data provide clear evidence for a sex difference in the functional organization of the brain for language and indicate that these variations exist at the level of phonological processing.

We studied neurologically normal right-handed males (mean age 28.5 years) and females (mean age 24.0 years). Subjects performed four distinct same-different tasks on visually displayed stimuli: line judgement, letter case, rhyme and semantic category. The decision (same versus different) and response components (pressing a response bulb for same pairs) of these tasks are comparable, but there is a difference in the type of linguistic information engaged by each. In the line-judgement task, subjects viewed two sets of four lines with right or left orientations, one above the other, and determined whether the upper and lower displays had the same pattern of left/right alternation

(engaging visual information processing). In the letter-case judgement task, two sets of consonant strings were displayed, and subjects determined whether they contained the same pattern of case alternation (engaging both visual and orthographic processing). In the rhyme-judgement task, subjects determined whether two nonsense word strings rhymed (engaging visual, orthographic and phonological processing: subjects must map the letter strings onto phonological representations). Finally, in the semantic category task, subjects determined whether two words came from the same semantic category (engaging visual, orthographic, phonological and semantic information). By subtracting the line from the case task, activation in regions of interest associated with orthography can be isolated; by subtracting the case from the rhyme task, phonological regions of interest can be isolated; and by subtracting the nonsense word rhyme from the semantic category task, regions of interest associated with lexical semantic processing can be isolated.

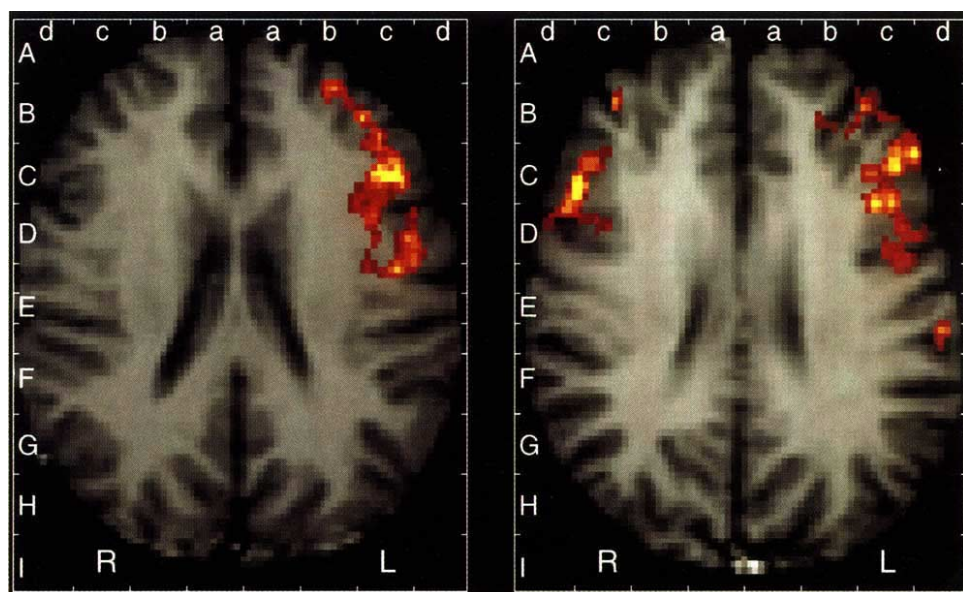
Selection of candidate regions of interest was motivated by previous neuropsychological and neuroimaging investigations of language function. Behavioural research on word recognition isolates two types of coding relevant to lexical identification: orthographic (pertaining to letter encoding) and phonological (pertaining to phoneme encoding)^{22,23}. Preliminary analysis identified one region uniquely associated with orthographic processing (extrastriate, ES). A second region, located within the superior aspect of the inferior frontal gyrus, roughly encompassing Brodmann's areas 44/45 (which we term IFG) and previously shown to be activated in speech tasks when phonetic decisions are required^{24,25}, was found to be uniquely associated with phonological processing on rhyme judgements. The rhyme-judgement task was also associated with activation at sites in both the superior temporal gyrus and middle temporal gyrus, areas that fall within traditional language regions. But the semantic task activated both of these areas significantly more strongly than the rhyme task, suggesting that these regions subserved both phonological and lexical semantic processing. The IFG, by contrast, was uniquely associated with phonological processing, and here we focus on the contrast between IFG and ES regions in examining sex differences.

A $2 \times 2 \times 3 \times 3$ analysis of variance (ANOVA) was performed with the following factors: region of interest (IFG versus ES), hemisphere (left versus right), task (case versus rhyme versus semantic), and sex (male versus female). For each subject, the number of pixels showing significant changes in magnetic resonance signal intensity was computed in the initial split *t*-test (Fig. 2) and these values were subsequently entered as the dependent measure in the ANOVA.

A significant sex-by-hemisphere interaction was observed: $F(1, 36) = 14.74$, $P < 0.001$. For males, the mean number of pixels activated were 11.7 and 5.0 for the left and right hemispheres, respectively; the corresponding values for females were 9.4 and 12. As shown in Fig. 1, activation during rhyming in males was lateralized to the left inferior frontal regions. In contrast, activation during this same task in females engaged this region

FIG. 1 Composite images of the distribution of activations comparing rhyme-case tasks (phonological processing) for 19 males (left image) compared to 19 females (right image). Colour dots represent pixels for which the mean value of the split *t*-statistic from averaging the 19 subjects was higher than 0.4 (dark red dots are close to 0.4; yellow approaches 1.0). The images were cluster-filtered so that isolated activated pixels without at least four activated neighbours were dropped. Images were coregistered using a piece-wise warping algorithm. Six image subregions were identified as described in Fig. 2 legend and each was linearly scaled so that the anatomic reference points (the anterior and posterior commissures and midline) and brain edges aligned. Coordinates²⁹ were then assigned to each region. Activations are shown for level 6–7 (*z* = 20) of the Talairach system²⁹. The Talairach reference grid has been superimposed on each image. Capital letters A–I (y-axis) and lower-case a–d (x-axis) designate the Talairach proportional grid system. R and L are right and left sides of brain, respectively. Sections are oriented with anterior portions at top of figure. Males show unilateral activation, primarily in the left inferior frontal gyrus (centred on coordinates *x* = 5.0, *y* = 1.8, *z* = 20), with minor activation of the left middle frontal gyrus. In females, phonological processing activates both the left (L) and right (R) inferior frontal gyri. There is smaller activation of the left and right middle frontal gyri (centred on coordinates *x* = 3.4, *y* = 4.5, *z* = 20) and of the left post-central gyrus (centred on coordinate *x* = 6.0, *y* = -2.1, *z* = 20).

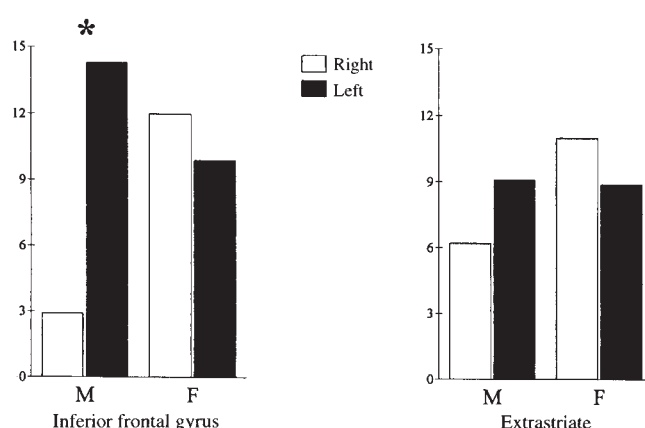
METHODS. Imaging was performed on a 1.5 Tesla GE 'Signa' MR imaging system equipped with echo-planar imaging (EPI) hardware from Advanced NMR (Wilmington). Conventional spin echo sagittal *T*₁-weighted (TE (echo time), 11 ms; TR (repetition time), 500 ms; FOV (field of view), 24 cm; slice thickness, 5 mm; slice gap, 2.5 mm; 256 × 128 × 1Nex (number of excitations)) localizer scans were first obtained from which axial-oblique activation images were



prescribed. Three axial-oblique slices, 8 mm thick, were obtained parallel to a line connecting the anterior and posterior commissures. The inferior slice was centred at Talairach 9, the middle slice at Talairach 7–8, and the superior slice at Talairach 6–7. Conventional spin echo images (TE, 11 ms; TR, 500 ms; FOV, 40 × 40 cm; 256 × 192 × 2 Nex) of these slice locations were collected before the start of each activation paradigm. These anatomic images, which are in exact registration with the activation images, were later used as the basis images on which to overlay activation maps. Subjects' heads were immobilized within the head coil by using a neck support, foam wedges and a restraining band drawn tightly around the forehead. The calculated *t*-maps showed no significant rim artefacts or apparent activation at strong edges, confirming that head movements were not significant.

FIG. 2 Three-way interaction between region of interest, hemisphere and sex. Ordinate represents mean activations across tasks for inferior frontal gyrus and extrastriate regions, respectively. Overall $F(1, 36) = 7.77$, $P < 0.01$. For females (F), the means for left (black bars) and right (grey bars) extrastriate were 8.9 and 11.0, and for left and right inferior frontal gyrus region were 12.0 and 10.0; these means were not significantly different. For males (M), the corresponding means were 9.1 and 6.2 for left and right extrastriate, and 14.3 and 2.9 for left and right inferior frontal gyrus region. The difference for males in the inferior frontal gyrus region is significant, $F(1, 18) = 22.34$, $P < 0.001$ (indicated by asterisk). To examine this three-way interaction further, the sex by hemisphere interaction was analysed for the two regions separately. The sex by hemisphere interaction was highly reliable in the inferior frontal gyrus region, $F(1, 36) = 20.90$, $P < 0.001$ but nonsignificant in extrastriate regions ($P > 0.05$). We further examined the ratio of right hemisphere to left hemisphere activation in the IFG. Eleven of 19 females but no males had a right to left hemisphere ratio ≥ 0.70 ; in fact for 9 of these 11 females the ratio was ≥ 1.0 . Thus, more than half of the female subjects produced strong bilateral activation in this region; by contrast, no males showed this pattern.

METHODS. Data analysis was performed using software written in MATLAB (Mathworks, Natick, MA). The activation images were collected using an EPI gradient echo sequence (flip angle, 60°; TE, 45 ms; TR, 1,500 ms; FOV, 40 × 20 cm; 128 × 128 × 1Nex) in the three slice locations described. Twenty-four images per slice location were collected while the subject performed one of the four (line, case, rhyme or semantic) activation tasks. Each task was run 4 times, with the order of successive tasks randomized, a total of 96 images per slice per task being collected. The first seven images from each series were dropped because they were obtained before a steady state of the echo-planar sequence was reached. The remaining seventeen images from each series were median-filtered. Before median filtering, the temporal mean intensity image was subtracted from each acquisition and added back after filtering. Subject head movements were analysed but not corrected. When movements larger than one pixel were found, those image data were discarded and only the unshifted data were analysed. There was no significant artefact from motion effects at the edges that could produce false activation in functional MRI. The activated pixels were detected for each pair of activation tasks using a split Student *t*-test. The split *t*-test divides the data into two parts and performs a separate *t*-test on each half dataset. If the *t*-value for a given pixel from both *t*-maps was above 2, the pixel was



considered to be activated. This analysis does not correct for any residual temporal correlation between successive images that can arise when the activation response varies during the task³⁰, but these corrections to our *t*-values are negligible for the steady-state response achieved during our experiments. For normally distributed data, $t > 2$ corresponds to $P < 0.05$. This threshold for activation provides a consistent criterion for identifying true activity from other sources of signal variation. On each anatomical image, the positions of the anterior commissure and posterior commissure and the direction of the midline were found manually. These reference points and the edges of the brain let us define the standard Talairach coordinate system for each subject. Each brain (anatomical image and activation map) was then rescaled to the standard Talairach form using cubic proportional fitting for each block defined by the anatomical landmarks. This procedure was remarkably successful; the major sulci and gyri can be clearly recognized on the composite image obtained by adding 38 Talairach-scaled anatomical images. Finally, each anatomical region of interest was identified in the Talairach coordinate system and approximated by a set of squares (Fig. 1). The number of activated pixels in each region was then used as a measure of the level of activation for any pair of tasks.

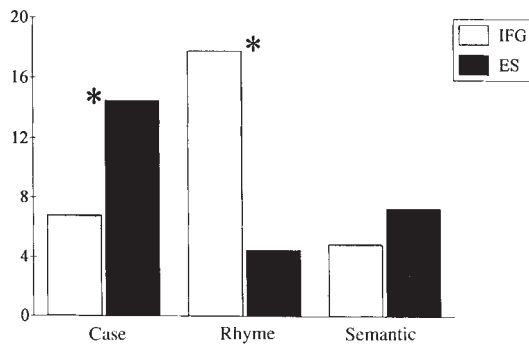


FIG. 3 Task by region of interest. Ordinate represents mean activations for case, rhyme and semantic subtractions in the inferior frontal gyrus (IFG; grey bars) and extrastriate (ES; black bars) regions, respectively. A significant interaction between task and region was observed, $F(2, 72) = 9.94$, $P < 0.001$. The means for the case, rhyme and semantic subtractions in the IFG region were 6.8, 17.8 and 4.9; corresponding means in the extrastriate region were 14.5, 4.5 and 7.3, respectively. Separate contrasts revealed that in the IFG region rhyme significantly differed from both case, $F(1, 36) = 10.0$, $P < 0.001$, and semantic, $F(1, 36) = 13.88$, $P < 0.001$, whereas case and semantic did not differ ($F < 1.0$). In the extrastriate region, case significantly differed from both rhyme $F(1, 36) = 8.37$, $P < 0.001$, and semantic, $F(1, 36) = 4.27$, $P < 0.05$. The rhyme and semantic conditions did not differ ($F < 1.0$). To test the hypothesis further that extrastriate areas subserve orthographic processing while the IFG region subserves phonological processing, we contrasted activation produced in a rhyme–case versus a rhyme–line subtraction. By the logic of the design, the former subtraction differs only in phonology whereas the latter subtraction differs in both orthography and phonology. A significant difference between these two subtraction conditions should therefore be observed in the extrastriate as only the rhyme–line should isolate orthography and there should be no difference in the IFG region as both conditions should isolate phonology. As expected, the effect was significant in the extrastriate area, $F(1, 36) = 17.89$, $P < 0.001$. The means were 4.5 and 19.0 for the rhyme–case and rhyme–line conditions, respectively. In the IFG region, the contrast was not significant ($P > 0.10$) with means of 17.6 and 23.1 for the rhyme–case and rhyme–line conditions, respectively. Asterisks indicate tasks that significantly differ between regions ($P < 0.001$).

bilaterally. Error rates on each task were extremely low (on average one error per 20 trials) and did not vary systematically with task or by sex, suggesting that the tasks did not differ significantly in their difficulty. The three-way interaction between region of interest, hemisphere and sex was significant ($F(1, 36) = 7.77$, $P < 0.01$) and is shown in Fig. 2. Activation in the IFG region was left-lateralized for males but bilateral for females, whereas extrastriate activation was bilateral for both males and females. In addition, these analyses confirmed that the case–line subtraction (which isolates orthographic processing) more strongly activates extrastriate sites whereas the rhyme–case subtraction (which isolates phonological processing) more strongly activates the IFG region (Fig. 3).

The regions of interest examined encompass those areas traditionally considered to be critical for language^{26–28}. We recognize, however, that our study does not provide information about every possible brain region and that there may be other sites relevant to phonological processing which may not show gender differences. Although we do not want to claim that phonological processing makes no demand on right hemisphere sites in males, we wish to emphasize that in a site uniquely serving phonological processing, the IFG, females devote greater right hemispheric resources to the task.

Our results indicate that it is now possible to isolate specific components of language and, at the same time, to relate these language processes to distinct patterns of functional organization in brain in neurologically normal individuals. Using this

strategy, we have demonstrated remarkable differences in the functional organization of a specific component of language, phonological processing, between normal males and females. Future studies designed to examine either gender differences in language function or the neural mechanisms related to language, for example, should be specific for the component of language assessed and determined in both males and females. □

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Integration of motion and stereopsis in middle temporal cortical area of macaques

David C. Bradley, Ning Qian* & Richard A. Andersen

Division of Biology (216-76), California Institute of Technology, Pasadena, California 91125, USA

THE primate visual system incorporates a highly specialized subsystem for the analysis of motion in the visual field^{1–6}. A key element of this subsystem is the middle temporal (MT) cortical area, which contains a majority of direction-selective neurons^{1–3}. MT neurons are also selective for binocular disparity (depth), which is perplexing given that they are not sensitive to motion through depth⁷. What is the role of disparity in MT? Our data suggest an important link between disparity and transparent motion detection. Motion signals in different directions tend to inhibit each other within a given MT receptive field⁸. This inhibi-

* Present address: Center for Neurobiology and Behavior, Columbia University, New York, NY 10032, USA.

tion has an averaging effect which minimizes MT responses to random motion signals created by light intensity changes and other non-motion stimuli (motion noise)⁹. But, in the absence of disparity cues, inhibition may also occur between surfaces moving in different directions through the same part of the visual field (transparent motion), thus impairing the detection of either surface. Here we show that inhibition in MT occurs mainly between motion signals with similar disparities. Transparent surface movements at different depths are thus represented independently in MT (that is, without inhibiting each other) whereas spurious motion signals from a given surface tend to cancel out. To our knowledge, these results provide the first evidence for a functional integration of motion and disparity in MT.

We used the stimuli shown in Fig. 1*b* and *c* to study the effect of binocular disparity on motion-opponent inhibition in MT. Each stimulus consisted of a 'preferred' random dot pattern, which moved in the preferred direction and at the preferred depth (disparity) for a given cell, and an 'antipreferred' pattern, which moved in the opposite direction and could assume a variety of depths. The preferred and antipreferred patterns were superimposed to form a transparent motion stimulus (Fig. 1*b*) in which opposite motions were interspersed. We also created two-panel, 'motion border' stimuli to study inhibition between movements in different parts of the receptive field (Fig. 1*c*).

Results were similar for the two stimulus types. In general, both suppressed MT firing rates relative to responses elicited by preferred-direction motion alone. In nearly two-thirds of the neurons, however, the amount of suppression depended on the depth of the antipreferred pattern (Figs 1 and 2). In many neurons (~40% of all tested), suppression was strongest when the antipreferred pattern was at or near the depth of the preferred pattern (Fig. 1). Thus, in these neurons the disparities that caused the strongest excitation in the preferred direction also caused the strongest inhibition in the antipreferred direction. We refer to these as 'planar opponent' cells. In other cells, suppression was strongest when the antipreferred pattern was either in front of or behind the preferred pattern ('near/far opponent' cells; about 15%). Finally, in a small percentage of cells, suppression was weakest when the opponent patterns were at similar depths ('additive' cells; about 4%). The remaining third of the neurons were strongly suppressed regardless of the depth of the

TABLE 1 Classification of MT neurons according to responses to transparent and motion border stimuli

	Number of cells (% modulation $\pm$ s.d.)			
	Planar opponent	Near/far opponent	Additive	No apparent tuning
Transparent stimuli (<i>n</i> = 127)	41 (49 $\pm$ 29%)	22 (48 $\pm$ 48%)	6 (38 $\pm$ 9%)	58 (NA)
Motion border stimuli (<i>n</i> = 124)	53 (41 $\pm$ 22%)	21 (47 $\pm$ 25%)	3 (27 $\pm$ 4%)	47 (NA)

In each stimulus, the preferred direction pattern was held at the cell's optimal disparity, whereas the antipreferred direction pattern assumed a variety of disparities. Cells were then classified according to the disparity tuning curve for the antipreferred direction (see for example, Fig. 1*b* and *c*). If this curve showed a well defined minimum within 0.2° of the cell's optimal disparity, the cell was classified as 'planar opponent'. If there was a well defined maximum within 0.2° of the optimal disparity, the cell was 'additive'. If these criteria were not met, but responses were imbalanced on either side of the optimal disparity (such that the antipreferred pattern produced stronger suppression in front of or behind the preferred pattern), the cell was termed 'near/far opponent'. Cells not clearly satisfying these criteria were said to be without apparent tuning. Data shown represent numbers of neurons in each class. Values in parentheses give the per cent modulation; that is, the range of responses due to different antipreferred disparities, normalized in each case to the response to a preferred pattern alone. Data shown are from two cerebral hemispheres in 2 rhesus monkeys. A total 146 MT neurons were studied with either transparent or motion border stimuli (about half with both stimulus types).

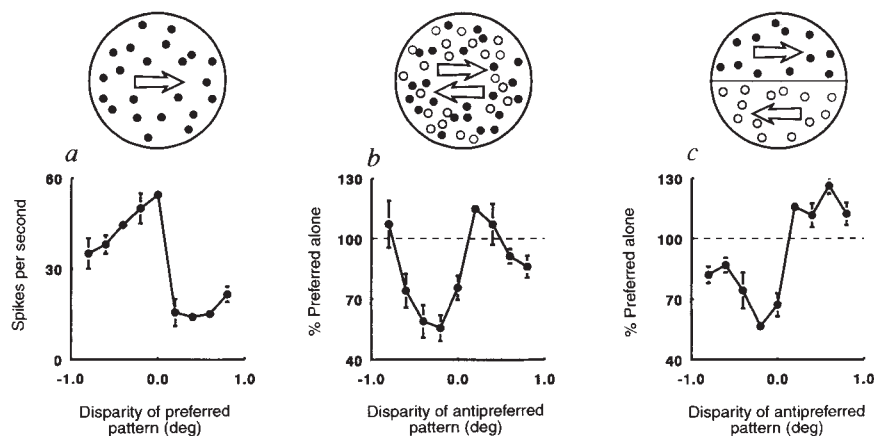
antipreferred pattern (Fig. 2*b, e*). The distribution of response types is detailed in Table 1.

Figure 2*a* and *d* shows averaged data for the planar opponent neurons. These cells were suppressed by ~40% (relative to preferred motion alone) when preferred and antipreferred patterns had the same disparity, and this suppression decreased by more than half to 10–20% (on average) when the patterns were separated by $\pm 0.8^\circ$. Most of the suppression could thus be relaxed

FIG. 1 Data from a 'planar opponent' neuron.

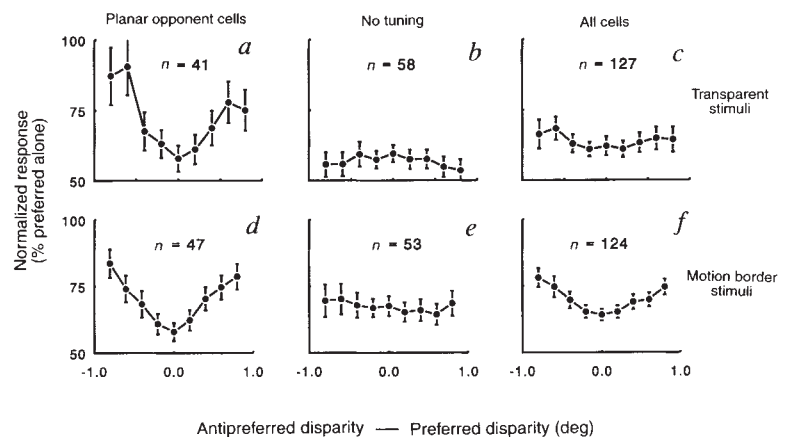
The schematics across the top show the stimuli. Left: a single random dot pattern moving in the preferred direction (in this case three o'clock); middle: two superimposed patterns moving in opposite directions (transparent stimulus); right: two adjacent patterns moving in opposite directions (motion border stimulus). Solid circles represent dots moving in the preferred direction; open circles represent the antipreferred direction. Stimuli were 4° diameter and dot density was 5 dots per cm². Dots were rendered in stereoscopic depth using colour separation⁴. Disparity tuning curves are shown below: for the preferred pattern alone (left), for the antipreferred pattern in a transparent stimulus (middle), and for the antipreferred pattern in a motion border stimulus (right). For the transparent and motion border stimuli, the preferred pattern was always at 0° disparity (the optimal disparity for this cell). Responses to the two-pattern stimuli were normalized to responses to a preferred pattern alone. The data show that disparity tuning for the antipreferred direction is roughly opposite to tuning for the preferred direction. Error bars give standard error of the mean.

METHODS. Male rhesus monkeys were trained to fixate a point of light while stimuli were presented in the desired portion of the visual field. Eye position was monitored with a scleral search coil¹⁹. MT neurons were accessed on long, dorso-ventral penetrations with tungsten



microelectrodes. After a neuron was isolated, its receptive field mapped, and its preferred direction and disparity determined, a series of motion-opponent stimuli were presented in the centre of the receptive field. Each stimulus lasted one second and was repeated (on average) 5 times; responses are expressed as the average firing rate over the last 800 ms of the stimulus periods. Additional experimental details may be found elsewhere^{8,9}.

FIG. 2 Average responses to transparent and motion border stimuli. Graphs show firing rates (normalized to responses to preferred motion alone) as a function of the difference between preferred and antipreferred disparities (reflecting the apparent depth between the opponent surfaces). Top row: data for transparent stimuli. Bottom row: data for motion border stimuli. Left column: average responses for cells classified as 'planar opponent' (Table 1). Middle column: average responses for cells without apparent suppression tuning (Table 1). Right column: average responses for all cells. Values are means $\pm$ standard error. The planar opponency trend seen in the population data for motion border stimuli (f) was highly significant ($P < 0.001$; linear regression of normalized firing rate versus absolute antipreferred-preferred disparity difference). For the planar opponent cells (left column), the average tuning width for half-maximal effect (estimated graphically) was 1.0° for transparent stimuli and 0.9° for motion border stimuli.



by placing the opponent patterns at different depths. In fact, more than a third of the planar opponent cells (14/42 for transparent stimuli, 30/53 for motion border stimuli) did not respond significantly better to a single, preferred pattern than to an opponent stimulus with preferred and antipreferred patterns at different depths ($P > 0.05$, t -test). Thus in these cells inhibition depended strongly on the disparity associated with the antipreferred direction. However, we also found a sizeable group of cells in which inhibition did not vary with antipreferred disparity (Fig. 2b, e), suggesting that the planar opponent neurons represent a distinct group. When the data from all neurons are considered together (Fig. 2c, f), the planar opponent trend remains visible, at least in the motion border data (Fig. 2f) where the trend was highly significant ($P < 0.001$; see legend for details).

Our data thus show that motion-opponent inhibition in MT tends to occur within a given disparity channel; that is, between motion signals from similar stereoscopic depths. This finding suggests a physiological basis for recent psychophysical observations on the role of disparity in transparent motion perception¹⁰. Motion detection thresholds are elevated for opponent motion stimuli such as those used here¹¹⁻¹³, and it has been shown that coherent motion perception is almost completely suppressed if dots are 'paired' so that each small spatial region contains two opposite motions¹⁰. But when these paired stimuli contain disparity cues, such that opponent motions occur in different depth planes, observers readily perceive two moving surfaces: that is, transparent motion¹⁰. These findings are closely paralleled by our present data, which show strong MT suppression by opponent patterns at similar depths but little or no suppression by stimuli incorporating a disparity differential (Fig. 2a, d). These observations, taken with other studies linking MT activity to motion perception¹⁴⁻¹⁶, suggest that disparity cues facilitate transparent motion detection by limiting inhibition in MT between motion signals from different visual depths.

Transparent motion detection might seem simpler if opponent inhibition did not occur in the first place. Directional V1 neurons in the macaque in fact show much less inhibition⁸ and might thus seem better suited than MT neurons for detecting transparent motion. But because of the absence of opponency, V1 neurons respond well to motion noise⁹ and therefore do not reliably report coherent motion. Most MT neurons, on the other hand, respond best to coherent motion⁹ and continue to do so under transparent conditions as long as disparity (or other) information is sufficient to dissociate individual surface movements. Thus, by integrating motion and disparity cues, MT succeeds in representing transparent motion while retaining essential noise reduction properties.

It should be clarified that even without disparity cues, the opponent stimuli used here appear to be transparent; that is, two independently moving surfaces are perceived. One might infer from this that disparity cues are not important in trans-

parent motion perception, given that transparency is already detectable without them. We must re-emphasize, however, that motion detection, measured psychophysically, is in fact substantially impaired for these and other opponent stimuli¹⁰⁻¹³. Moreover, we showed that motion perception is only possible in such stimuli because of small regions containing unbalanced (unopposed) motion signals¹⁰. If these unbalanced regions are prevented, coherent motion perception is lost entirely¹⁰. Under such conditions, the importance of disparity, which is sufficient to render even these balanced stimuli fully transparent, is unequivocal. We therefore believe that the effects of disparity elucidated here contribute to an important mechanism for transparent motion detection.

Our data provide evidence for a function for binocular disparity in MT. In an early demonstration of disparity tuning in MT⁷, it was found that individual MT neurons do not respond selectively to motion in stereoscopic depth (although population coding cannot be excluded). The role of disparity in MT has since remained unclear. Perhaps disparity allows a more detailed description of moving objects; that is, depth and velocity rather than simply velocity. However, all the visual qualities of moving objects need not be represented in MT simply because they are moving; other aspects, such as colour and texture, could be represented in other cortical areas^{17,18}. Why should disparity be represented in MT? Our data suggest that disparity plays an intrinsic role in MT motion computation by providing a means of distinguishing motion signals from different surfaces. Like disparity, other surface characteristics, such as speed, colour and spatial frequency, may also facilitate transparent motion detection by placing constraints on opponent inhibition in MT. □

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Synchronized oscillations in interneuron networks driven by metabotropic glutamate receptor activation

Miles A. Whittington*, Roger D. Traub†† & John G. R. Jefferys*§

* Neuronal Networks Group, Department of Physiology and Biophysics, St Mary's Hospital Medical School, Imperial College, London W2 1PG, UK

† IBM Research Division, T. J. Watson Research Center, Yorktown Heights, New York 10598, USA

†† Department of Neurology, Columbia University, New York, New York 10032, USA

PARTIALLY synchronous 40-Hz oscillations of cortical neurons have been implicated in cognitive function. Specifically, coherence of these oscillations between different parts of the cortex may provide conjunctive properties^{1,2} to solve the 'binding problem': associating features detected by the cortex into unified perceived objects. Here we report an emergent 40-Hz oscillation in networks of inhibitory neurons connected by synapses using GABA_A (γ-aminobutyric acid) receptors in slices of rat hippocampus and neocortex. These network inhibitory postsynaptic potential oscillations occur in response to the activation of metabotropic glutamate receptors. The oscillations can entrain pyramidal cell discharges. The oscillation frequency is determined both by the net excitation of interneurons and by the kinetics of the inhibitory postsynaptic potentials between them. We propose that interneuron network oscillations, in conjunction with intrinsic membrane resonances and long-loop (such as thalamocortical) interactions, contribute to 40-Hz rhythms *in vivo*.

Oscillations at ~40 Hz occur in CA1 pyramidal cells in hippocampal slices following the excitatory response to afferent stimulation applied 2 s after a conditioning train (which activates metabotropic glutamate receptors³ and erodes slow inhibition⁴; Fig. 1a). The oscillation frequency is independent of membrane potential in the impaled pyramidal cell over the range -40 to -90 mV (data not shown). Application of the GABA_B-receptor antagonist 2-hydroxysaclofen greatly prolongs the oscillations (Fig. 1b), which have power spectra peaking at ~40 Hz (Fig. 1d). Although the components of the oscillation appear to be depolarizing, they are fast inhibitory postsynaptic potentials (i.p.s.ps), reversed by the effects of the conditioning train⁵: they are blocked by the GABA_A-receptor antagonist bicuculline (Fig. 1c), and they survive the block of ionotropic glutamate receptors and GABA_B receptors, a treatment that isolates fast i.p.s.ps^{4,6} (Fig. 2). Power spectra of responses evoked in CA1 by glutamate application under these conditions reveal a strong peak close to 40 Hz and a small peak at double that frequency (Fig. 2b). Rhythmic i.p.s.ps occur synchronously in pairs of pyramidal cells separated by ~1 mm (Fig. 2a), with cross-correlations revealing strong interactions with phase lags of 1.0–3.5 ms (Fig. 2c). When pyramidal cells are held close to threshold by current injection, the i.p.s.p. oscillation elicited by glutamate modulates their discharge into a collective 40-Hz rhythm synchronized between cells ~1 mm apart (Fig. 2e).

The 40-Hz oscillations are a collective behaviour of the network of interneurons in the hippocampus. First, the inhibitory postsynaptic currents (i.p.s.cs) are large, up to 10 times those expected for the activity of a single interneuron⁷. Second, individual components often have a complex waveform (Fig. 2d). Third, they are synchronized between pyramidal cells separated

by 1 mm (Fig. 2a). Fourth, they are evoked by stimuli that do not elicit monosynaptic i.p.s.cs (see Fig. 4C).

The necessary features for the inhibitory network oscillation were examined using computer simulations of a network of 128 realistic hippocampal interneurons (Fig. 3a). The simulated network oscillates at ~40 Hz as long as the interneurons are interconnected by GABA_A-ergic synapses and receive tonic excitation, which is provided in the experiments by glutamate or the metabotropic glutamate receptor agonist (1S, 3R)-1-aminocyclopentane-1,3-dicarboxylic acid ((1S, 3R)ACPD). Gap junctions⁸ are not required, but, if present, enhance synchronization. In contrast, gap junctions appear central to a model of one form of synchronization induced by 4-aminopyridine in pharmacologically isolated hippocampal interneurons⁹ (simulations: R.D.T., unpublished).

The key role of mutual inhibition in determining the frequency of the 40-Hz i.p.s.c. oscillation is shown by the effect of modify-

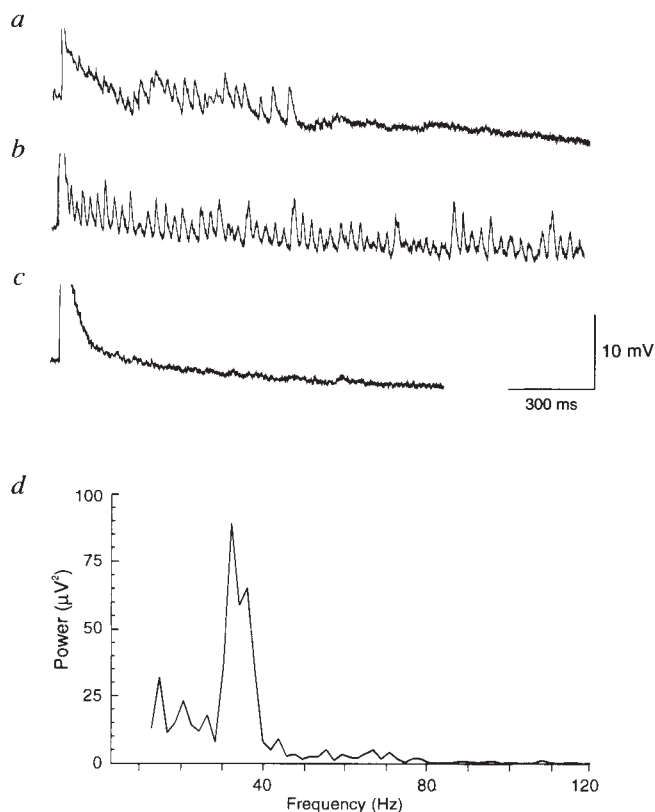


FIG. 1 40-Hz oscillations in intracellular recordings from CA1 pyramidal cells, evoked by afferent stimulation 2 s after a weak conditioning train in the absence of any drugs (a), are prolonged by the presence of the GABA_B antagonist 3-amino-2-(4-chlorophenyl)-2-hydroxypropylsulphonic acid (2-OH-saclofen) (b), and abolished by the GABA_A antagonist bicuculline (c). The response to afferent stimulation without the conditioning train is an e.p.s.p. as seen in c, followed by fast and slow i.p.s.ps. The power spectrum of the last 1.8 s of the response in b reveals a sharp peak at 33.4 Hz (d).

METHODS. Intracellular recordings from CA1 pyramidal cells from 400-μm-thick slices of dorsal hippocampus maintained at 36 °C and perfused with artificial cerebrospinal fluid (ACSF, in mM: NaCl, 135; NaHCO₃, 16; CaCl₂, 2; KCl, 3; NaH₂PO₄, 1.25; MgCl₂, 1; glucose, 10) under warm, wet, 95% O₂, 5% CO₂. In b, ACSF also contained 0.2 mM 2-OH-saclofen and in c, 10 μM bicuculline methiodide. The conditioning train consisted of stimuli at half the strength needed to evoke an action potential, repeated at 100 Hz for 1 s. Sharp glass micropipettes were filled with 2 M potassium methylsulphate (resistance 30–60 MΩ). Cross-correlation and power spectrum analyses used SPIKE2 (Cambridge Electronic Design, UK).

§ To whom correspondence should be addressed.

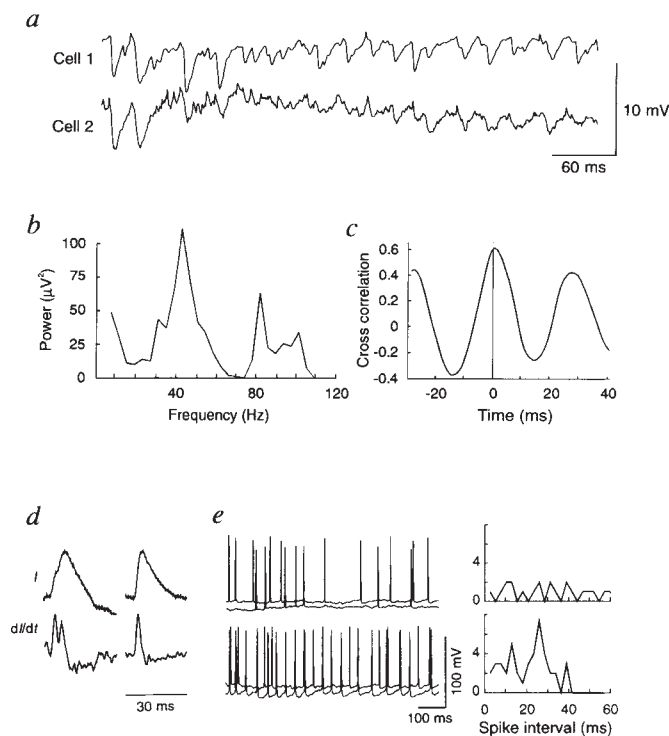
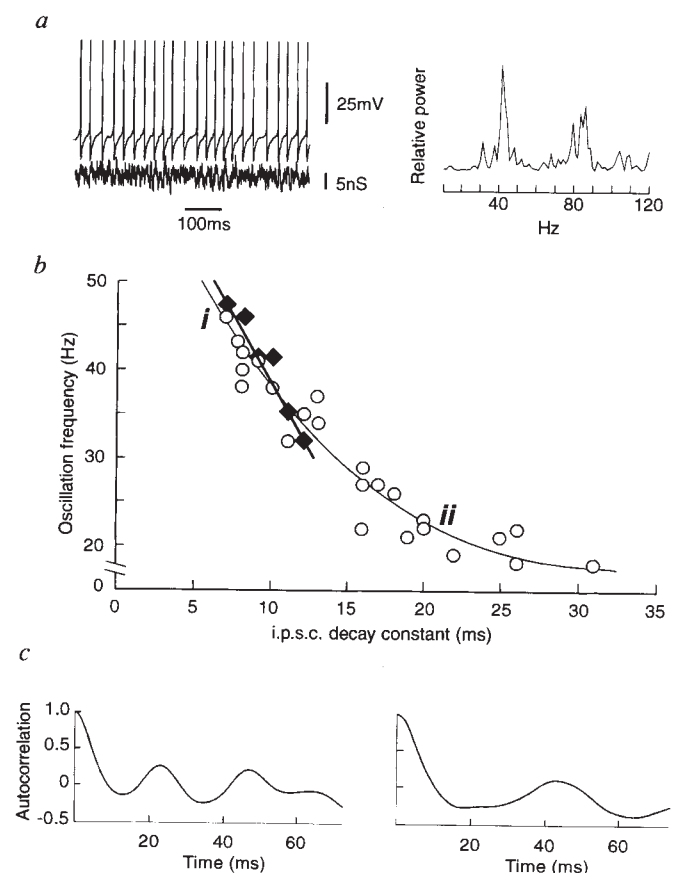


FIG. 2 40-Hz oscillations in CA1 pyramidal cells with blockade of ionotropic excitatory postsynaptic potentials (e.p.s.ps) and slow i.p.s.ps. *a*, A pair of pyramidal cells, 1 mm apart, were depolarized to -40 mV by current injection in bridge mode. Pressure ejection of glutamate into stratum pyramidale generated trains of i.p.s.ps synchronously in the two cells. *b*, Power spectrum of a train of i.p.s.ps in a single pyramidal cell indicating a strong peak at 42 Hz along with a harmonic at 82 Hz. *c*, Cross-correlation of the trains of i.p.s.ps in *a* revealed a strong correlation between the cells, with cell 2 lagging by 1.5 ms, and a 37-Hz rhythm (27-ms period). *d*, Voltage-clamp records of individual i.p.s.ps in a 40-Hz train as in *a* often had complex rise and decay kinetics, markedly different from the simple kinetics more typical of unitary i.p.s.cs. *e*, top, Current injection (0.6 nA) into a pair of pyramidal cells (resting potentials -67 and -69 mV) elicited action potentials independently in each cell, and accommodation in both (not shown). Frequency distribution of interspike intervals for the combined cells was flat. Bottom, Current injection concurrently with pressure ejection of glutamate produced: less accommodation, partial synchrony of the action potentials and combined output from the 2 cells having a modal interspike interval of 26 ms (38 Hz) during the plateau phase of depolarization. **METHODS.** Intracellular recordings from CA1 pyramidal cells from 400- μ m-thick slices in the presence of drugs to block AMPA and NMDA receptor-mediated e.p.s.ps and slow i.p.s.ps (20 μ M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 100 μ M D(-)-2-amino-phosphonovaleric acid (D-APV) or 50 μ M ketamine, and 2-OH-saclofen. Glutamate, 100 μ M, was applied by pressure ejection through a blunt micropipette in stratum pyramidale (0.6 kg cm^{-2} , 100 ms). Recordings of trains of i.p.s.ps at -40 mV were done with electrodes containing an additional 50 mM QX314. *b*, *c*, *e*, Cross-correlation and power spectrum analyses used SPIKE2 (Cambridge Electrode Design, UK). The i.p.s.cs were recorded using switched single-electrode voltage clamp (Axoclamp).

FIG. 3 Frequency of inhibitory neuronal network oscillations is a function of i.p.s.c. kinetics in individual inhibitory interneurons. *a*, Simulation of a network of 128 interneurons interconnected by GABA_A synapses. Left, Somatic potential (spikes truncated) and total synaptic conductance to the same neuron (a measure of population activity). Right, Power spectrum of conductance signal of the same neuron. Note peaks at ~ 40 and 80 Hz. *b*, Responses were recorded from electrophysiologically identified inhibitory neurons evoked by glutamate application (Fig. 2) during the wash-in of 2 μ M pentobarbital. These reveal the relationship of the principal oscillation frequency, from autocorrelation analyses, versus the decay constant (τ_D) of individual i.p.s.cs within a train (derived from single exponential curve fit; $\circ$). Note the close match with the relationship between spectral peak and τ_{GABA} predicted by computer simulation (thick line, $\blacklozenge$). The nonlinearity of the experimental data at large τ_D is a function of the dual effects of pentobarbital in increasing both τ_{GABA} and i.p.s.c. amplitude. *c*, left, Autocorrelations of i.p.s.c. trains in control solution reveal a period of 22.7 ms (44 Hz); τ_D was 9.1 ± 0.4 ms ($n=20$ epochs before pentobarbital; *b*; *i*). Right, During equilibration with pentobarbital the period slowed to 44.5 ms (22 Hz) at *ii* in *b*; τ_D reached >30 ms ($n=30$ epochs). **METHODS.** Simulations: individual neurons: each model neuron has 46 somadendritic (SD) and 5 axon initial segment compartments, with SD $\tau_m=37.5$ ms and somatic $R_{\text{input}}=96.5 \text{ M}\Omega$. In response to tonic depolarizing current injection, the cells generate repetitive trains of action potentials, with rapid after hyperpolarizations (AHPs) and little adaptation. The proximal and middle dendrites contain voltage-dependent sodium conductance in order to replicate the data of ref. 28 that a single dendritic release site can evoke a short-latency action potential (R.D.T. and R. Miles, manuscript in preparation). Network interactions: we simulated 128 neurons interconnected by 'GABA_A' synapses, with 20 connections per neuron, and no other types of interaction (such as GABA_B, electrical synapses). Synapses are located on 7 different proximal dendritic compartments, with one release site per connection, and reversal potential of -15 mV from resting potential. Unitary conductance time course consists of a sudden rise to 2.0 nS, followed by exponential decay with τ_{GABA} , a parameter from 6 to 12 ms. The cells receive a tonic somatic bias current of 0.225 to 0.255 nA, different for each cell. Simulations were done on an IBM SP1 parallel computer using 16 nodes. A 1,000-ms simulation took about 85 min. Experimental: inhibitory neurons impaled in stratum oriens of CA1 were identified by their brief action potential (<3 ms at base) and lack of accommodation. The decay phase of i.p.s.cs clearly separated from their neighbours



were fitted by a single exponential (EPC package, Cambridge Electronic Design Ltd); control τ_D was in the range 8–15 ms, faster than that for pyramidal cells (22.4 ± 0.8 ms). After equilibration with 2 μ M pentobarbital the decay of i.p.s.cs was too slow for reliable measurements.

ing i.p.s.c. duration. Computer simulations of an inhibitory network in which i.p.s.c. decay time course varies from 6 to 12 ms predict a close relationship with frequency (Fig. 3b, thick line). This is confirmed by recordings of the response of interneurons to glutamate during the wash-in of 2 μ M pentobarbital (Fig. 3b, thin curve). The change in slope at decay constants >12 ms probably reflects the effect of higher doses of pentobarbital on i.p.s.c. amplitude, which is not included in the simulation.

Single electric stimuli evoke i.p.s.cs oscillating at 40 Hz when ionotropic glutamate receptors and GABA_B receptors are blocked (Fig. 4A). Such stimuli were effective whether delivered close to the recording, or whether far enough away not to produce a monosynaptic i.p.s.c. (Fig. 4B, C). Tetrodotoxin (TTX) abolishes the oscillation, leaving either a monosynaptic i.p.s.c. or no response⁶.

Drugs in the bath blocked ionotropic glutamate receptors, so that metabotropic glutamate receptors are candidates to drive the oscillation of the inhibitory network. The metabotropic receptor antagonist (+)- α -methylcarboxyphenylglycine (MCPG) blocks i.p.s.c. oscillations evoked electrically, but has no effect on monosynaptic i.p.s.cs (Fig. 4D). The agonist, (1S, 3R)-ACPD, elicits 40-Hz i.p.s.c. oscillations in CA1 pyramidal cells very similar to those evoked electrically, which are also blocked by MCPG (Fig. 4E, F). (Oscillating i.p.s.ps in response to metabotropic agonists have been described before,

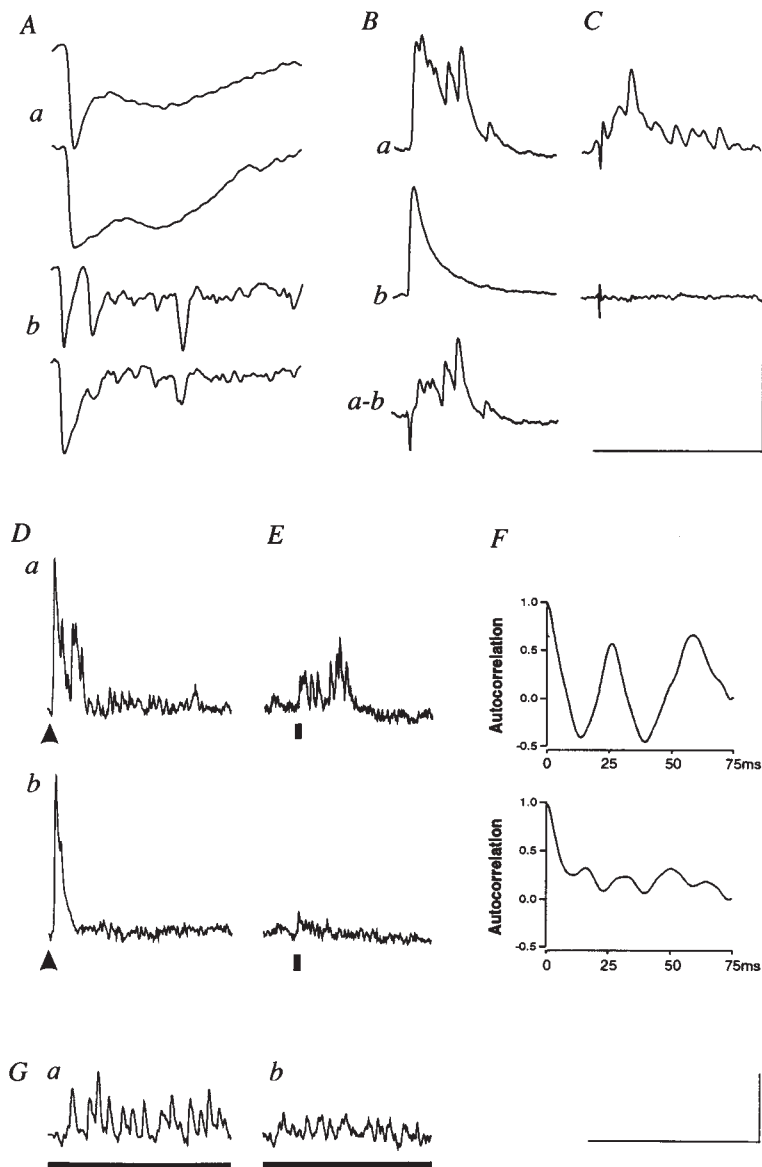
but attributed to rhythmic activity in single interneurons³.) Tetanic stimulation of the kind required for long-term potentiation (LTP) provides the necessary conditions for these 40-Hz oscillations¹⁰.

Application of (1S, 3R)-ACPD to neocortical slices results in prolonged i.p.s.c. oscillations at ~40 Hz, which are most prominent in superficial laminae (Fig. 4G). They have properties very similar to those studied in detail in the hippocampus and probably reflect a similar mechanism.

Cortical oscillations at 30–50 Hz became a central issue for cortical mechanisms of perception when they were described in discharges of groups of neurons and in local field potentials in cat visual cortex^{11,12}. Their synchrony over wide distances, and between hemispheres^{13–15}, provides a potential solution for the problem of grouping, or 'binding' features detected at widely separate cortical regions into perceived objects. They also provide means for scene segmentation to distinguish which features belong to which objects¹⁶. In man, 40-Hz reverberating activity occurs between thalamus and cortex^{17,18}. Similar frequencies occur in other parts of the brain, including the hippocampus during theta activity¹⁹ and the olfactory system, where they were first described^{20,21}.

Several theories have been proposed for the cellular mechanism of 40-Hz oscillations². Intrinsic ~40-Hz oscillations occur in sparsely spiny stellate¹ and pyramidal cells^{1,22} in neocortex,

FIG. 4 Activation of metabotropic glutamate receptors in the absence of GABA_B-receptor-mediated inhibition is required for sustained interneuronal network oscillations. Aa, Simultaneous recordings of biphasic i.p.s.ps in CA1 pyramidal cells evoked by proximal stimulation in the presence of 20 μ M CNQX and 50 μ M D-APV. Responses were evoked from depolarized membrane potentials of -42 and -40 mV for the two cells and show an initial fast GABA_A, followed by a slow GABA_B, receptor mediated potential. Ab, Simultaneous recordings from the same cells after the addition of 0.2 mM 2-OH-saclofen to block GABA_B receptors reveal stimulus-evoked synchronous trains of i.p.s.ps. Ba, Voltage-clamp records of i.p.s.c. trains evoked by proximal stimulation as in Ab (holding potential -40 mV). Bb, After the addition of 2 μ M tetrodotoxin to block action potentials i.p.s.cs evoked in the same cell were monophasic⁴, suggesting that the i.p.s.c. train (subtraction a-b) depended on an intact network. Ca, Distal stimulation (CA3c) demonstrating that synaptic excitation from remote sites can elicit i.p.s.c. oscillations in area CA1 without an initial large monosynaptic component. Cb, Addition of 2 μ M TTX blocked all evoked activity. D, Metabotropic glutamate receptors are necessary for i.p.s.c. oscillations. Da, Proximal stimulation of stratum pyramidale ($\blacktriangle$) generated a maximal i.p.s.c. in a CA1 pyramidal cell followed by oscillations. Db, Bath application of 0.2 mM (+)- α -methylcarboxyphenylglycine (MCPG) did not affect the initial i.p.s.c. but almost completely abolished oscillations. Ea, i.p.s.c. oscillations lasting 200 ms were elicited by pressure ejection of (1S, 3R)-ACPD (100 μ M 1.2 kg cm⁻², 10 ms) ($\blacksquare$). Eb, Bath application of MCPG abolished oscillations induced by (1S, 3R)-ACPD. Fa, Autocorrelation analyses of responses to pressure ejection of (1S, 3R)-ACPD in E revealed a period of 27 ms (37 Hz). Fb, No coherent oscillations were seen in the presence of 0.2 mM MCPG. G, Pressure ejection of (1S, 3R)-ACPD into coronal neocortical slices cut in planes either including the rostral or the caudal extremities of the hippocampus also elicited oscillating i.p.s.cs in the presence of APV, CNQX and 2-OH-saclofen as described above. Those in superficial laminae (Ga; layer II, 37 Hz) were more prominent than those in deeper laminae (Gb; layers IV/V, 35 Hz). Scale bars A, 15 mV, 200 ms; B, C, 2 nA, 200 ms; D, E, G, 1 nA, 500 ms.



and in thalamocortical projection neurons²³. Two hypotheses^{21,24} were proposed for the population ~40-Hz rhythms in the olfactory cortex; EEG power spectra, and phase relations with unit recordings, supported negative feedback between populations of excitatory and inhibitory neurons over the coupling of intrinsically oscillatory neurons^{21,24}.

The data presented here suggest a new model. We propose that networks of inhibitory neurons can generate 40-Hz oscillations in pyramidal cells that can entrain their firing^{25,26}. Emergence of network oscillations would be assisted by the intrinsic properties of interneurons¹. Entrainment will be particularly robust if the excitatory cells are themselves intrinsic

oscillators^{1,22}. Longer-range synchrony could occur through coupling of the inhibitory oscillator by intercortical interconnections or, in the case of the neocortex, by resonating with the thalamocortical loop²³ (but see ref. 2). The inhibitory network oscillator is under synaptic control: it is blocked by slow, GABA_B-receptor-mediated i.p.s.ps, and driven by tonic activation of inhibitory neurons, here by metabotropic glutamate receptors, which have potent, excitatory effects on interneurons^{3,27}. These synaptic inputs provide the means to determine which sets of interneurons generate synchronized 40-Hz i.p.s.ps *in vivo*, and hence which populations of pyramidal cells will have their activity entrained. □

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The E-selectin-ligand ESL-1 is a variant of a receptor for fibroblast growth factor

Martin Steegmaler*, Agneta Levinovitz*†, Sandra Isenmann*, Eric Borges*, Martin Lenter*, Hans P. Kocher‡, Beate Kleuser‡ & Dietmar Vestweber*§

* Hans-Spemann-Laboratory at the Max-Planck-Institute for Immunobiology, D-79108 Freiburg, Germany

‡ Sandoz Pharma Ltd, CH-4002 Basel, Switzerland

E-SELECTIN is an inducible cell-adhesion molecule on endothelial cells, which mediates the binding of neutrophils and functions as a Ca²⁺-dependent lectin^{1–3}. We have recently identified a 150K glycoprotein as the major ligand for E-selectin on myeloid cells, using a recombinant antibody-like form of mouse E-selectin as an affinity probe^{4,5}. Here we report the isolation of a mouse complementary DNA for this E-selectin ligand (ESL-1). The predicted amino-acid sequence of ESL-1 is 94% identical (over 1,078 amino acids) to the recently identified chicken cysteine-rich fibroblast growth-factor receptor⁶, except for a unique 70-amino-acid amino-terminal domain of mature ESL-1. Fucosylation of ESL-1 is imperative for affinity isolation with E-selectin-IgG. A fucosylated, recombinant antibody-like form of ESL-1, but not of L-selectin, supports adhesion of E-selectin-transfected Chinese hamster ovary cells. Antibodies against ESL-1 block the binding of mouse myeloid cells to E-selectin. ESL-1, with a structure essentially identical to that of a receptor, thus functions as a cell adhesion ligand of E-selectin.

To obtain sequence information about the 150K E-selectin ligand, we purified the ligand from the mouse neutrophilic pro-

genitor 32Dcl3. Peptides were generated and microsequencing of four of them revealed strong homology to the recently identified chicken cysteine-rich fibroblast growth-factor (FGF) receptor (CFR)⁶, with homologies of 83, 92, 100 and 100%. CFR is completely unrelated to any of the other known FGF receptors⁷. A reverse-transcribed polymerase chain reaction (RT-PCR) fragment was used to screen a cDNA library generated from poly(A)⁺ RNA of 32Dcl3 cells. The cDNA clone with the largest open reading frame covered the sequence depicted in Fig. 1. The complete amino-acid sequence of the mature ESL-1 protein was 94% identical to the chicken CFR except for a 70-amino-acid domain at the N terminus, replacing the first 35 amino acids of the N terminus of CFR. This was confirmed by a second cDNA clone which covered the first 507 base pairs (bp) (coding for the first 167 amino acids) of the depicted sequence and which was isolated from another, specifically primed cDNA library. A signal sequence of 27 amino acids is predicted, leaving a mature polypeptide of 1,148 amino acids. This sequence predicts a transmembrane protein with a relative molecular mass (*M_r*) of 131K, consisting of a 1,114, amino-acid extracellular domain, a 21-amino-acid transmembrane region and a short, charged 13-amino-acid cytoplasmic tail. The five putative N-glycosylation sites and the calculated *M_r* of the mature, unglycosylated protein backbone correspond well with the determined *M_r* of the purified E-selectin ligand before (150K) and after (135K) treatment with endoglycosidase F^{4,5}. These data suggest that the ligand is only weakly, if at all, modified by O-linked carbohydrate side chains.

Chinese hamster ovary (CHO) cells were transfected with the ESL-1 cDNA alone or in combination with a cDNA coding for the human fucosyl transferase III (FT-III)^{8,9} and analysed in immunoprecipitations with rabbit antiserum against the purified E-selectin ligand from 32Dcl3 cells. The serum recognized a protein of 150K already in mock-transfected as well as in FT-III-transfected CHO cells (Fig. 2a). In ESL-1-transfected, as well as in double-transfected CHO cells, this immunoprecipitation signal was strongly enhanced. As shown in sequential immunoprecipitations (Fig. 2b), the endogenously expressed 150K protein of CHO cells did not bind to E-selectin IgG, but became competent to bind to E-selectin if FT-III was expressed in these

† Present address: Department of Pathology, Huddinge University Hospital, S-14186 Huddinge, Sweden.

§ To whom correspondence should be addressed.

CAG AAT

1Pmet Ala Val Cys Gly Arg Val Arg Gly Met Phe Arg Leu Ser Ala Ala Leu Pro Leu Leu Leu Leu Ala Ala Ala Gly Ala Gln Asn

94 GGT CAC GGT CAG GGC CAG GGT CCG ACC AAC TTT GGG CCC TTC CCA GCG AAA GGT GGA GGC GGC AGC CCG CGC GCC CAA CAG CCG OCT CAG
308 Gly Hie Gly Glu Glin Gly Glu Pro Gly Thr An Phie Gly Pro Phie Gly Glu Glu Glu Glu Glu Glu Glu Glu Ser Pro Ala Gly Glin Glu Pro Glu Gin

187 CAG CCT CAG TTA TCG CAG CAG CAG CAG CCG CCG OCT CAG CAG CAG CAG CAG CAA CAG CAG CAG TCG CTG TTG GCG GCG GCG GCG CTC CCG
611 Glu Pro Glu Leu Ser Glu Glin Glin Glin Glu Pro Pro Glu Glin Glin Glin Glin Glin Glin Glu Ser Leu Phie Ala Ala Gly Gly Leu Lye

280 CCG CCG GCG GCG GCG GCG GCG CCG GGT GCG ACT GCG GSA GGC TGG AAG CTG GCG GCG GAA GAG TCC TCG CCG GAG GAG GTG ACC OCT GTC TGC
921 Ala Arg Arg Gly Gly Ala Gly Pro Gly Gly Thr Gly Gly Gly Trp Lys Leu Ala Glu Glu Glu Ser Cys Arg Glu Asp Val Thr Arg Val Cys

373 CCC AAA CAC ACC TGG AOC AAC AAC CTG CGC GTG CTC Glu GUG TGC CCG CAG GGT AGG GAG CCC GAG AAC CAG ATT TCT TCA CAG TCC AAT CAT
1273 Pro Lys Hie Thr Trp Ser An An Leu Ala Val Leu Glu Cys Leu Glu An Asp Val Arg Glu Pro Glu An Glu Ile Ser Ser Asp Cys An His

466 TTG TTG TGG AAT TAC AAG CTG AAC CTG ACT ACT GAT CCC AAA TTT GAA TOT GTG CCG AGG GAG GTT TCG AAG TCA ACT ATT TCC GAG ATT AAA
1549 Leu Leu Trp An Tyr Lys Leu An Leu Thr Thr Asp Pro Lys Phie Glu Ser Val Ala Arg Glu Val Cys Lys Ser Thr lle Ser Glu lle Lys

559 GAA TGT GCG GAA GAA CCA GTT GGA AAA GGT TAC ATG GTT TCC TCG TTG GTG GAT CAT CGA GGC AAC ACT ACT GAG TAT CAG TGT CAT CAA TAC
1859 Gly Hie Cys Ala Glu Glu Pro Val Gly Lys Gly Tyr Met Val Ser Gly Met Val Ser Gly Glu Glu Val Ser Cys Arg Gly An lle Thr Glu Tyr Glu Cys Hie Glin Thr

652 ATT ACC AAG ATG ACC GCG ATC ATT TTC AGT GAT TAT OCT TGC ATG CTC GGC TTC ATG GAT GAT TCC AAA AAT GAT ATC AAC CTC TTG AAA TGT
2169 Lle Thr Lys Met Thr Ala lle lle Phie Ser Asp Tyr Arg Glu lle Cys Gly Phie Met Asp Asp Cys Lys An Asp lle An Leu Leu Lys Cys

745 GCG AGC ATT OCT CTG GGG AAA AAG GAT GCA CAT TCA CAA GGT GAG CTG GTA TCA TGT TTG GAA AAA GCG CTG GTA AAG GCA GAA GAA AAA
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838 GAG CCG AAG ATT CAA GTC TCA GAA CTC TCG AAG AAA GCT ATT CTT AGG GTG GGT GCG GAT GCG TCC TOG GAT GAT TTT CAT TTA GAC CCG CAT TTT
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931 TAT TTT OCT TCG CAG GAT CAT GCG GCG OCT TTT TGC GAN AAT ACA CAA GGT GGT GAA GGA AGA ATP GAT TAT AAA TGC CTA TTT AAC CAT AAG TTT
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1024 GAA GAA TCC ATG AGT GAA AAG TGT AGA GAA CCA CTA ACT ACT AOC CAG AAG CTC ATT GGC CAG GAT TAT AAA GTC AGT TAC TCA TTA GGC AAA
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1303 ACC CCG GAG ATC ATC CTG AGC TOT GSA GCG GAG ATT GAA CAC CAT TGT TOT GTP ATA CAT CCG AAA GCG GSA ACC CTC CAC TGT CTG ATG AAA
4339 Ser Pro Glu lle lle Lys Ser Cys Arg Gly Glu lle Glu Hie Hie Cys Ser Gly Leu Hie Arg Lys Gly Arg Thr Leu Hie Cys Leu Met Lys

1396 GTG GTT CCG GGT GAA AAG GGG AAC CTT GGA ATG AAC TGC CAA CAG CCG GTT CAG CCA CTG ATT CAG GAG ACT CAG OCT GGT CCA CAG TAC CCG
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1489 ATT GAT CGA GCT TTG AAT GAA GCT TGT GAG TCT GTA ATA CAG GCG CTC AAA CAC ATA CGA TCC GGA GAC CCA ATG ATT CTC TCA TGT CTG
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1582 ATG GAG CAT TTA TAC AAG AAG ATG GTG GAA CAG TST GAA CCG CCG CTC TTA GAG CTA CAG TAT TTT ATC TCT GGT GAT TGS AAG TTG CAG
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1768 GTG TTT TCT TCG CTA TACAGA CAT GCG TAC CCG ACA GAA GAG CAA GSA AGG ACG CTC TCA CGA GAA TGT CGA GCT GAA GTC CAG AGS ATC CTG
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1954 GCG CAG CAG CTT GAG TGC CTT CAG CAC CAT CTC GAT GCA TGA GGT GTG GAA TGC AGA GAG ATC GTG GCG AAC CTC ACC GAG TTA GAG TCC GAG
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2047 GAT ATA CAA ATA GAA GCT TTG CTG ATG AGA GCG TST GAG OCT ATC ATT CAG AAC TTT TCC CAC GAT GTG GCA GAC AAC CAG ATC GAC TCT GSG
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2140 CAG CTG ATG GAG TGT CTG ATC CAG AAC CAG AAC CAG AAC CAG AAC TGT GCG ATT GCG CTT ACT CAC TTC CAG CTG GTA CAG ATG
7129 Asp Leu Met Glu Cys Glu Thr lle Glu An An Lys Hie Glu Lys Asp Met An Glu Lys Asp Met An Glu Lys Asp Met An Glu Lys Asp Met

2233 AAG GAT TTT CGA TTC TCT TAC AAG TTC AAA ATG GCG TCC AAG GAG GAT GTG TTA AAG CTT TCC CCC AAC ATA AAA AAG AAG GTG CAC GTA GTG
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2326 ATC TCC CTG AGC ACC ACT GTC CCG AAC GAC ACT GTG CAG GAG GCG AAG CAG CAG CCA GTA TCA CTT AAG TCC CAG CAG CTT GGT GTG GAA
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2419 GAG CTG GAG ATG AGA CAG GAG ATC CST TTG GAA CCA GAT CTG TAT GAA GCG TCC AAG AGT GAT CAG AAC TAC TGT TCT ACA GTG CAA TAT
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2512 GGA AAT GCT CAG ATT ATT GAA TGT CTA AAA GAA AAC AAG AAG CAG CTG AGT ACC GGT TSC CAG CAG AAA GTA TTT AAG CTG CAG GAG ACN GAG
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2605 ATG ATG CAG CCA GAG CTA CAG TAT ACT CTG ATG AGA GCT TGC AAG CAG ATG ATT AAG CCG TTC TGT CCA GAA GCA GAT TCT AAA ACT ATT TTG
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2698 CAG TGT TTA AAA CAA AAT AAG AAC AGT GAA TTG ATG GAT CCC AAA TGC AAA CAG ATG ATA ACC AAG CCG CAG ATC ACC CAG AAC ACA GAT TAC
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2791 CCG TTA AAC OCT GTG CTA AGS AAG GCG TGT AAA GCT GAG ATT OCT AAG TTC TGT CAT GGC ATC CTC AAC AAG GCT AAG GAG GAT TCA GAG CTA
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2884 GAA GCG CAA GTT ATC TCA TCG CTC AAG CTS AGA TAT GCT GAG CCG CCG TCC TCA CAG TGT GAG CAC CAG ATC CST ATC ATC ATC CAG GAG
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2977 TCT OCT CTG GAT TAC CCG CTG GAG OCT CAG CTC CAG CTC CAG TCC TCA GAT GAT ATT CCG AAT CTA TGT GCT GAA GAA CCA GCG CAG GAG
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3070 CAA CCA GCG CAA GTG GAA GAG TCC CTG AAG CTC AAC CTG CTC AAG ATC AAG CAA GAG CTG TGT AAA AAG GAA GTG TTA AAC ATC TTG AAG GAA
10229 Glu Thr Gly Glu Val Glu Glu Cys Leu Lys Val An Leu Leu Lys lle Lys Thr Glu Leu Cys Lys Lys Glu Val Leu An Met Leu Lys Glu

3163 AGC AAA CCA GAG ATC TTT GTG CAG CCG GTT CTT CAG ACA CCA TGT CCG TTG CAG ATT AAA CAG CAG TGT CCA GCG ATC ACC OCT GCG CCG GGY
10539 Ser Glu Lys Ala Asp lle Phie Val Asp Pro Val Leu Hie Thr Ala Cys Ala Leu Asp lle Lys Hie Hie Cys Ala Ala lle Thr Pro Gly Arg Gly

3256 OCT CAG ATG TCC TCC CTG ATG GAG CCG CTC GAA GAT AAG CGA GTG AGA TTG CAG CCA GAG TSC AAA AAG CSC CTC AAT GAC CCG ATT GAG ATG
10849 Arg Glu Met Ser Cys Leu

FIG. 1 Sequence of ESL-1. nucleotide and deduced amino-acid sequence of ESL-1. The putative signal peptide¹⁹ and transmembrane sequence are indicated by a heavy line. The four peptide sequences obtained by microsequencing are underlined. Potential N-linked glycosylation sites are indicated by —N—. The unique 70-amino-acid domain of ESL-1, for which no correlate is found in CFR, is boxed.

METHODS. 32Dcl3 cells (2×10^9) cultured as described⁴, were lysed in 150 ml of 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM CaCl_2 , 1% Triton X-100 for 10 min at 4 °C. The lysate was centrifuged twice at 20,000g for 15 min and the supernatant was incubated with 150 μl Protein A-Sepharose loaded with 550 μg of E-selectin-IgG (produced as described²⁰) for 6 h at 4 °C. Beads were washed four times with 50 mM Tris, pH 7.4, 400 mM NaCl, 1 mM CaCl_2 , 0.05% Triton X-100, once with the same buffer omitting the CaCl_2 , twice with 50 mM ammonium acetate, pH 7.1, 0.05% Triton X-100. Beads were eluted with 1 mM EDTA, 30 mM ammonium acetate, pH 7.1, and the eluted material was lyophilized. 20 μg of the ligand from 10 such preparations was electrophoresed in polyacrylamide gels, stained by Coomassie blue and digested with the protease LysC. Peptides were separated by HPLC, and four peptides were sequenced in a Beckman PI 2020 amino-acid sequenator. On the basis of the strong homology of these peptides to the chicken CFR, the following degenerated oligonucleotides were chosen as primers for RT-PCR: 5'-GCACCTAG-TGA(AG)AA-(GA)ATGGT(GACT)GA(GA)GAC-(CT)TG(CT)-3' and 5'-GCCTC-GAG(GA)TC(GAT)ATCAT(GA)-CA(CT)TT(GA)TC(CT)TG-3'. RT-PCR was done with poly-(A)⁺RNA which was isolated from 32Dcl3 cells as described²¹. The resulting 321-bp PCR fragment was used to screen a λ ZAPII library made from oligo-dT-primed cDNA of 32Dcl3 cells using the Stratagene cDNA synthesis kit. Out of nine isolated cDNA clones, one contained the complete, depicted nucleotide sequence. A second library was made from the same RNA using a

► mixture of random hexamers and a specific primer annealing between bp 483 and bp 507. Seven cDNA clones were isolated after screening with a PCR fragment covering the sequence between bp 318 and 459. One of these cDNA clones covered the 5' end of the depicted sequence up to bp 507, verifying that the 70-amino-acid N-terminal domain of the mature ESL-1 protein is indeed unrelated to the N terminus of the chicken CFR, despite the strong homology of the rest of the sequence. Sequencing was done on both DNA strands by the dideoxy method using plasmids generated by the Stratagene ExoMung deletion kit. The alignment of the protein sequences of mouse ESL-1 and chicken CFR was generated using the program PILEUP from the UWGCG²². The sequence appears in the GenBank/EMBL sequence databases under the accession number X84037.

cells. Similarly, the transfected ESL-1 bound to E-selectin IgG only if coexpressed with FT-III. Thus, fucosylation of ESL-1 is essential for binding to E-selectin. Fucosylated ESL-1 from double-transfected CHO cells was not precipitated by P-selectin IgG (Fig. 2c), demonstrating the E-selectin specificity of this ligand. A 150K glycoprotein could be immunoprecipitated with the anti-ESL-1 serum from many non-myeloid mouse cell lines, such as fibroblasts (L-cells), colon carcinoma (CMT), endothelioma (bEnd.3), teratocarcinoma (PCC4), lymphoma (L1-2) and myeloma (J558; SP2/0) (not shown). However, in none of these cases could the protein be affinity isolated with E-selectin IgG. We conclude that the ESL-1 protein (or the putative exact mouse

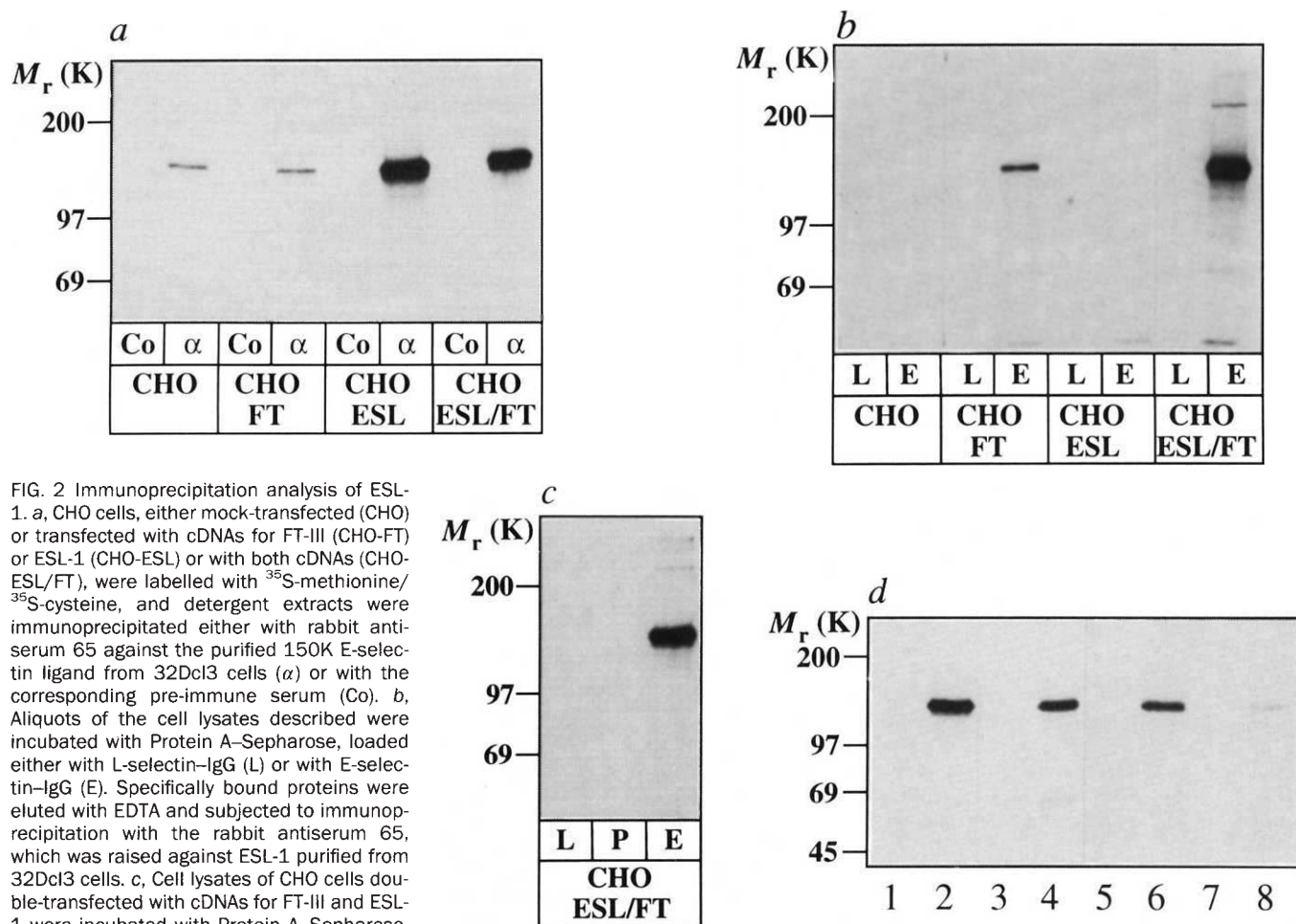


FIG. 2 Immunoprecipitation analysis of ESL-1. **a**, CHO cells, either mock-transfected (CHO) or transfected with cDNAs for FT-III (CHO-FT) or ESL-1 (CHO-ESL) or with both cDNAs (CHO-ESL/FT), were labelled with ³⁵S-methionine/³⁵S-cysteine, and detergent extracts were immunoprecipitated either with rabbit anti-serum 65 against the purified 150K E-selectin ligand from 32Dcl3 cells (α) or with the corresponding pre-immune serum (Co). **b**, Aliquots of the cell lysates described were incubated with Protein A-Sepharose, loaded either with L-selectin-IgG (L) or with E-selectin-IgG (E). Specifically bound proteins were eluted with EDTA and subjected to immunoprecipitation with the rabbit anti-serum 65, which was raised against ESL-1 purified from 32Dcl3 cells. **c**, Cell lysates of CHO cells double-transfected with cDNAs for FT-III and ESL-1 were incubated with Protein A-Sepharose, loaded either with L-selectin-IgG (L), P-selectin-IgG (P) or E-selectin-IgG (E). Specifically bound proteins were eluted and analysed as described under **b**. **d**, 32Dcl3 cells were labelled as described above and aliquots of the detergent extract were incubated with Protein A-Sepharose loaded with pre-immune rabbit IgG (lane 1), affinity-purified rabbit antibodies against the ESL-IgG fusion protein (lane 2), human IgG1 (lane 3) or E-selectin-IgG (lane 4). After washing, bound proteins were eluted either by boiling in SDS sample buffer (lanes 1 and 2) or by incubating with EDTA (lanes 3 and 4). Aliquots of the material eluted from the E-selectin-IgG matrix were subjected to a second immunoprecipitation with the rabbit pre-immune serum 89060 (lane 5), the rabbit anti-serum 89060 against ESL-IgG (lane 6), the rabbit pre-immune serum 96 (lane 7) or the rabbit anti-serum 96 against the C-terminal 13-amino-acid peptide of ESL-1 (lane 8). Lanes 1–4 and lanes 5–8 represent two different gels. Proteins were separated on 6% (**a**, **b** and **c**) or 8% (**d**) SDS-PAGE under reducing conditions and visualized by autoradiography. Molecular mass markers (in K) are indicated on the left.

METHODS. Immunoprecipitations were done as described⁵. Transfection of CHO cells (DUKX-B1, obtained from P. Vassalli) was done by electroporation (960 μ F; 250 V; 0.4 cm cuvettes) with the full-length cDNA for ESL-1 and/or the cDNA for FT-III^{8,9} (provided by J. Lowe) in the expression plasmid pcDNA1-Neo (Invitrogen, San Diego, CA). Stable

expression was achieved by selection with G418 using the neomycin resistance gene. Three rabbit antisera were raised against ESL-1. Anti-serum 65 was raised against 23 μ g of the 150K E-selectin ligand, which was purified from 32Dcl3 cells as described in Fig. 1. Anti-serum 96 was raised against a peptide covering the C-terminal 13 amino acids of ESL-1. Using an additional N-terminal cysteine, the peptide was conjugated to ovalbumin as described²¹. Anti-serum 89060 was raised against a fusion protein consisting of the complete extracellular part of ESL-1 including the last lysine residue next to the putative transmembrane region, fused to the Fc-part of the human IgG₁ sequence as described²⁰. The fusion protein was coexpressed with FT-III (in pcDNA1-Neo) in CHO cells and purified from the culture supernatant with Protein A-Sepharose. For affinity purification of the rabbit antibodies, the serum was first cleared of antibodies against the Fc-part of human IgG₁, using human IgG₁ conjugated to CNBr-Sepharose as adsorbant. Antibodies specific for ESL-1 were purified from the residual serum using the ESL-IgG fusion protein conjugated to CNBr-Sepharose as described²⁰. The L-selectin-IgG fusion protein contained the complete extracellular part of mouse L-selectin including the asparagine reset to the membrane and was expressed and purified as described for the ESL-IgG fusion protein. The E-selectin-IgG and P-selectin-IgG fusion proteins have been described²⁰.

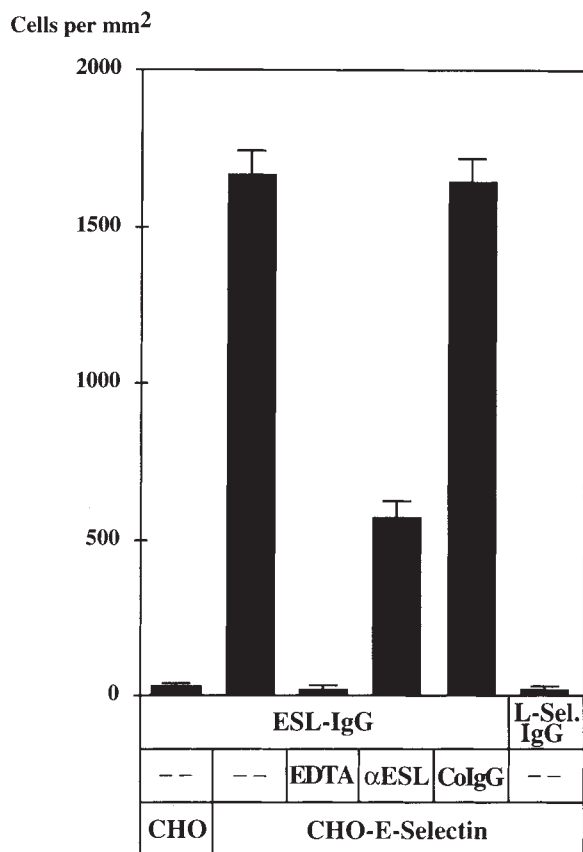


FIG. 3 E-selectin transfected cells bind to ESL-1. Cell adhesion assays were done with mock-transfected CHO cells (CHO) or E-selectin-transfected CHO cells (CHO-E-selectin) in 96-well microtitre plates coated with $10 \mu\text{g ml}^{-1}$ ESL-IgG or L-selectin-IgG (as indicated). Before the addition of cells, the fusion protein-coated wells were incubated with Hank's buffered salt solution (HBSS) (—), HBSS containing 1 mM EDTA (EDTA), $60 \mu\text{g ml}^{-1}$ antibodies from rabbit antiserum 89060 affinity-purified on ESL-IgG (αESL) or $60 \mu\text{g ml}^{-1}$ antibodies from the same serum, affinity-purified on human IgG₁ (ColgG). Unbound antibodies were washed away before the cells were added to the wells. The adhesion assay was done, and bound cells were quantified as described^{5,20}. ESL-IgG and L-selectin-IgG were both purified from FT-III-transfected CHO cells.

equivalent of the chicken CFR) is broadly distributed, whereas the glycoform of ESL-1, which is able to bind to E-selectin, is specific for myeloid cells (32Dcl3, HL60, mouse polymorphonuclear neutrophils (PMNs))⁵.

Affinity-purified antibodies of an antiserum against an ESL-1-immunoglobulin fusion protein (ESL-IgG) recognized the 150K ligand in immunoprecipitations from metabolically labelled 32Dcl3 cells (Fig. 2d, lane 2). In sequential immunoprecipitations the 150K glycoprotein ligand was affinity-isolated with E-selectin-IgG (Fig. 2d, lane 4), eluted with EDTA and subjected to immunoprecipitations with the antiserum against ESL-IgG and an antiserum against a peptide comprising the 13 amino acids of the cytoplasmic tail of ESL-1. Both antisera recognized the affinity-isolated 150K ligand (Fig. 2d, lanes 6 and 8).

We examined whether the ESL-IgG fusion protein coated onto plastic could support adhesion of E-selectin transfectants. Because the ESL-IgG fusion protein was purified from FT-III-transfected CHO cells, an L-selectin-IgG fusion protein, also purified from FT-III-expressing CHO cells, was used as control protein. E-selectin-transfected CHO cells, but not mock-transfected CHO cells bound, in an EDTA-sensitive manner, to ESL-IgG, whereas no binding was observed to L-selectin-IgG (Fig. 3). Binding was blocked by affinity-purified antibodies against the ESL-IgG fusion protein. No effect was seen with antibodies against the Fc-part of human IgG₁ purified from the same serum.

We tested whether these adhesion-blocking antibodies against ESL-1 would also be able to block adhesion of 32Dcl3 cells to plastic-coated E-selectin-IgG. Preincubating the cells with the affinity-purified antibodies clearly blocked adhesion (Fig. 4a), whereas no inhibitory effect was observed if the cells were preincubated with monoclonal antibody MEL-14 against L-selectin (Fig. 4a), which is strongly expressed on these cells⁴. Similarly,

the anti-ESL-1 antibodies partially blocked adhesion of 32Dcl3 cells (Fig. 4b) and of mouse PMNs (Fig. 4c) to TNF- α -activated sEnd.1 mouse endothelioma cells. Binding of PMNs was more efficiently blocked by anti P-selectin than by anti E-selectin antibodies. Therefore, the inhibitory effect of anti-ESL-1 antibodies was tested while simultaneously blocking P-selectin. Under these conditions anti ESL-1 blocked PMN binding with almost identical efficiency as anti-E-selectin antibodies.

Treatment of 32Dcl3 cells with trypsin (0.5 mg ml^{-1} for 15 min at 37°C) partially blocked cell binding to E-selectin-IgG (to 38%) whereas binding to P-selectin-IgG was completely blocked (not shown), reflecting the difference in protease sensitivity of E- and P-selectin ligands on HL60 cells¹⁰.

A variety of glycoproteins have been reported to support binding of E-selectin-transfected cells¹¹⁻¹⁷. Because binding of myeloid cells was only partially blocked by anti-ESL-1 antibodies, additional ligands for E-selectin cannot be excluded. However, none of the additional ligands (except PSGL-1) could be affinity-isolated with E-selectin-IgG. Our data demonstrate that ESL-1, which was identified as the major ligand on myeloid cells by affinity-isolation with E-selectin-IgG^{4,5}, mediates adhesion of myeloid cells to E-selectin.

In addition, the strong sequence homology of most of the ESL-1 molecule with CFR raises the question whether ESL-1 could function as a receptor. The unique 70-amino-acid N-terminal domain of ESL-1 suggests that ESL-1 may be a splicing variant of the mouse CFR-equivalent. Because E-selectin mediates the initial cell contact between leukocytes and endothelial cells, a step which is followed by the activation of leukocyte integrins, it is intriguing to speculate that the E-selectin ligand, ESL-1, may combine the function of a cell adhesion molecule with that of a receptor, triggering signals that are involved in

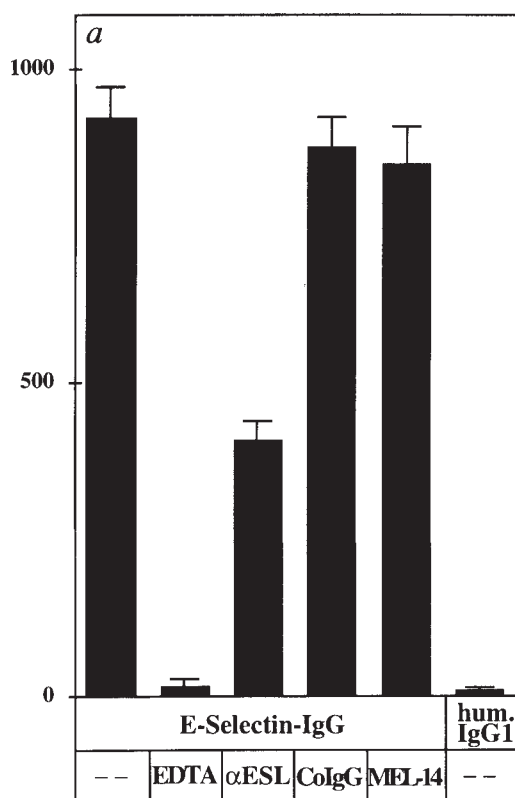
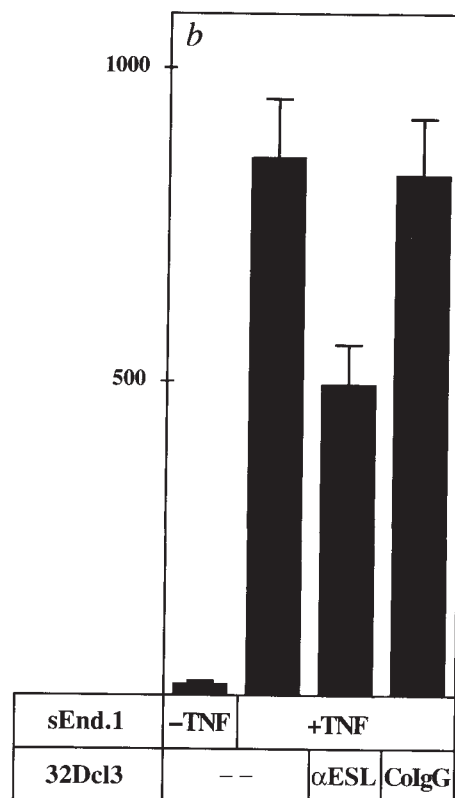
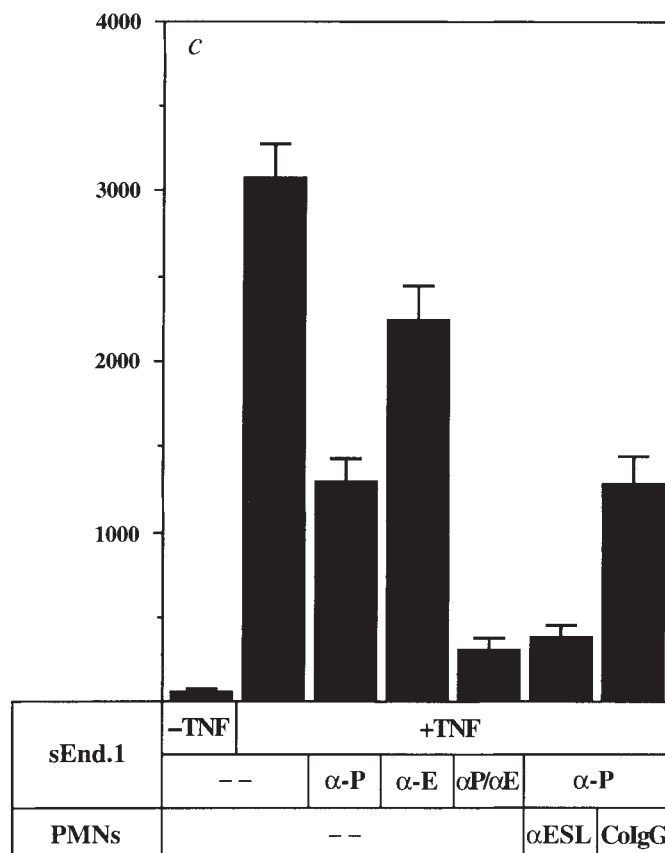
Cells per mm²Cells per mm²Cells per mm²

FIG. 4 Antibodies against ESL-1 block the binding of 32Dcl3 cells to E-selectin. **a**, Cell-adhesion assays were done with 32Dcl3 cells in 96-well microtitre plates coated with 4 $\mu\text{g ml}^{-1}$ of E-selectin-IgG or human IgG₁ (as indicated). Cells were preincubated with HBSS (—), HBSS containing 1 mM EDTA (EDTA), 60 $\mu\text{g ml}^{-1}$ antibodies from rabbit antiserum 89060, affinity-purified on ESL-IgG (αESL), 60 $\mu\text{g ml}^{-1}$ antibodies from the same serum affinity-purified on human IgG₁ (ColgG), or 60 $\mu\text{g ml}^{-1}$ monoclonal antibody MEL-14 against mouse L-selectin (MEL 14). **b**, Cell adhesion assays were done with 32Dcl3 cells by incubation with the monolayer of non-activated (–TNF) or TNF- α -activated (+TNF) sEnd.1 mouse endothelioma cells²³. 32Dcl3 cells were preincubated either with HBSS (—), HBSS containing 60 $\mu\text{g ml}^{-1}$ antibodies from rabbit antiserum 89060 against ESL-IgG, affinity purified on ESL-IgG (αESL), or HBSS containing 60 $\mu\text{g ml}^{-1}$ antibodies from the same serum affinity-purified on human IgG₁ (ColgG). **c**, Cell adhesion assays were done with mouse PMNs by incubation with the monolayer of non-activated (–TNF) or TNF- α -activated (+TNF) sEnd.1 mouse endothelioma cells. sEnd.1 cells were preincubated either with HBSS (—), hybridoma supernatant of the anti-mouse P-selectin rat IgG₁ antibody RB40.34 ($\alpha\text{-P}$), HBSS containing 250 $\mu\text{g ml}^{-1}$ of the anti-mouse E-selectin rat IgM antibody 21KC10 ($\alpha\text{-E}$) or the same concentration of 21KC10 in RB40.34 supernatant ($\alpha\text{-P}/\alpha\text{-E}$). PMNs were preincubated either with HBSS (—), HBSS containing 60 $\mu\text{g ml}^{-1}$ antibodies from rabbit antiserum 89060 against ESL-IgG, affinity-purified on ESL-IgG (αESL), or HBSS containing 60 $\mu\text{g ml}^{-1}$ antibodies from the same serum affinity-purified on human IgG₁ (ColgG). In all cases, unbound antibodies were washed away before the cells were added to the wells. The following procedures and reagents were as described: isolation of mouse PMNs⁴; adhesion assays and their quantification^{5,20}; the anti-mouse P-selectin mAb RB40.34²⁴ and anti-mouse E-selectin mAb 21KC10²⁰.

the activation process. Indeed, evidence has been reported that soluble E-selectin can induce activation of PMNs¹⁸. Whether ESL-1 is involved in this process remains to be tested. □

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Selective requirement for MAP kinase activation in thymocyte differentiation

Jose Alberola-Ila^{*†}, Katherine A. Forbush^{*†},
Rony Seger^{‡§}, Edwin G. Krebs^{‡§}
& Roger M. Perlmutter^{*†§¶}

Departments of ^{*} Immunology, [‡] Pharmacology, [§] Biochemistry, [¶] Medicine (Medical Genetics) and [†] Howard Hughes Medical Institute, University of Washington SL-15, Seattle, Washington 98195, USA

ENGAGEMENT of the T-cell receptor (TCR) with cognate ligands provokes different outcomes depending on the developmental stage of the T cell and on the properties of the ligand. In immature thymocytes TCR stimulation may result in maturation (positive selection) or death (negative selection), whereas in mature T cells it may induce proliferation, death or unresponsiveness^{1–5}. To investigate the different signals involved in these processes, we have analysed the role of the MAP kinase (MAPK) cascade, which is required for growth-factor-stimulated replication and for differentiation in other cell types^{6–9}, by expressing a catalytically inactive form of MAPK kinase (MEK-1) in thymocytes, thereby blocking MAPK activation. We find that positive selection of these cells is inhibited but that negative selection and TCR-induced proliferation are unaffected. Our results indicate that the intracellular signals regulating lineage commitment in T cells parallel those in photoreceptor cell specification in *Drosophila*¹⁰ and vulval cell differentiation in *Caenorhabditis elegans*¹¹, suggesting that general rules for cell-type specification could apply among all metazoans.

¶ Present address: Department of Membrane Research and Biophysics, The Weizmann Institute of Science, Rehovot 76100, Israel.

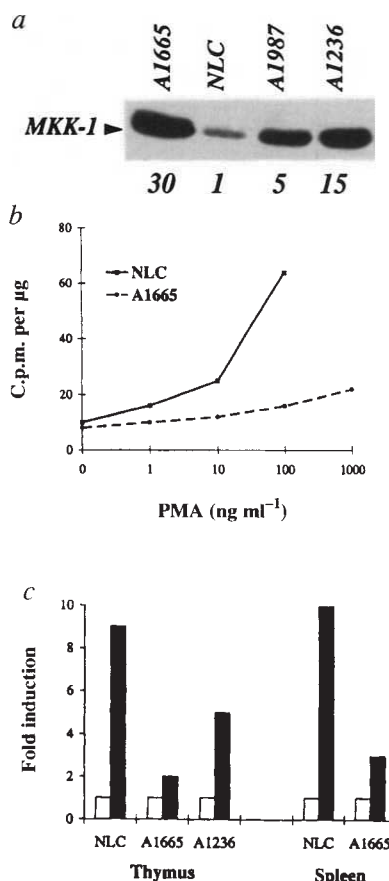


FIG. 1 Generation and characterization of *lck*-MEK-1_{A97} transgenic mice. **a**, Expression of MEK-1 in wild-type and transgenic thymocytes. Shown is an immunoblot made using 25 µg of total cellular protein comparing thymocytes from normal littermate controls (NLC) with thymocytes from three different transgenic lines identified by founder number. The relative abundance of immunoreactive MEK-1 protein, measured using serial dilutions, is indicated under each lane. **b**, Total thymocytes from A1665 or normal littermate control mice were isolated and stimulated for 10 min with different doses of phorbol myristyl acetate (PMA). Shown is the extent of activation of MAPK, as judged by phosphorylation of myelin basic protein. **c**, Dose-response relationship between MEK-1_{A97} protein expression and MAPK activation in thymocyte extracts. Activation of MAPK is still substantially inhibited in splenocytes from A1665 animals. ■, Treated with PMA; □, untreated. **METHODS.** The K97A mutation in MEK-1 (ref. 26) was generated by polymerase chain reaction (PCR) as described⁸, and the resulting cDNA was then cloned into the *Bam*HI cloning site of the p1017 vector²⁷. Purification and injection at 5 ng µl⁻¹ of the *Not*I fragment containing the *lck*-MEK-1_{A97} transgene into (C57BL/6J × DBA/2J) F₂ mouse zygote pronuclei was done according to ref. 28. Transgenic founders were detected by hybridization of genomic DNA with a human growth hormone (hGH) probe, and stable lines of mice were generated by backcrossing founders with C57BL/6J mice (Jackson Laboratories). **a**, Immunoblotting was performed as described in ref. 29. **b**, **c**, Total thymocytes or splenocytes were resuspended in HBSS and stimulated with PMA at different concentrations (**b**) or at 100 ng ml⁻¹ (**c**) for 10 min. Reactions were stopped with ice-cold HBSS. Cells were washed and lysed in buffer H⁸. MAPK was assayed on eluates from DE52 columns⁸ using myelin basic protein as substrate.

We generated transgenic mice that expressed MEK-1 bearing an alanine-for-lysine substitution at position 97 under the control of the thymocyte-specific *lck* proximal promoter (*lck*-MEK-1_{A97}). Levels of immunoreactive MEK-1 protein in thymocytes of these mice exceed those seen in control animals by 5- to 30-fold (Fig. 1a), and although the *lck* promoter functions poorly in mature T cells¹², a 10-fold elevation in MEK-1 protein abund-

ance persists in the splenic T lymphocytes of young adult *lck*-MEK-1_{A97} mice of the A1665 line. This high-level expression of MEK-1_{A97} protein effectively blocked MAPK activation, as revealed by measuring phosphorylation of myelin basic protein by partially purified MAPK from fractionated cell lysates, even after exposing thymocytes or splenic T cells to concentrations of phorbol esters that maximally stimulate MAPK in control cells (Fig. 1b, c). Similarly, we could not detect any MAPK activation in transgenic animals after crosslinking of the CD3/TCR complex (data not shown).

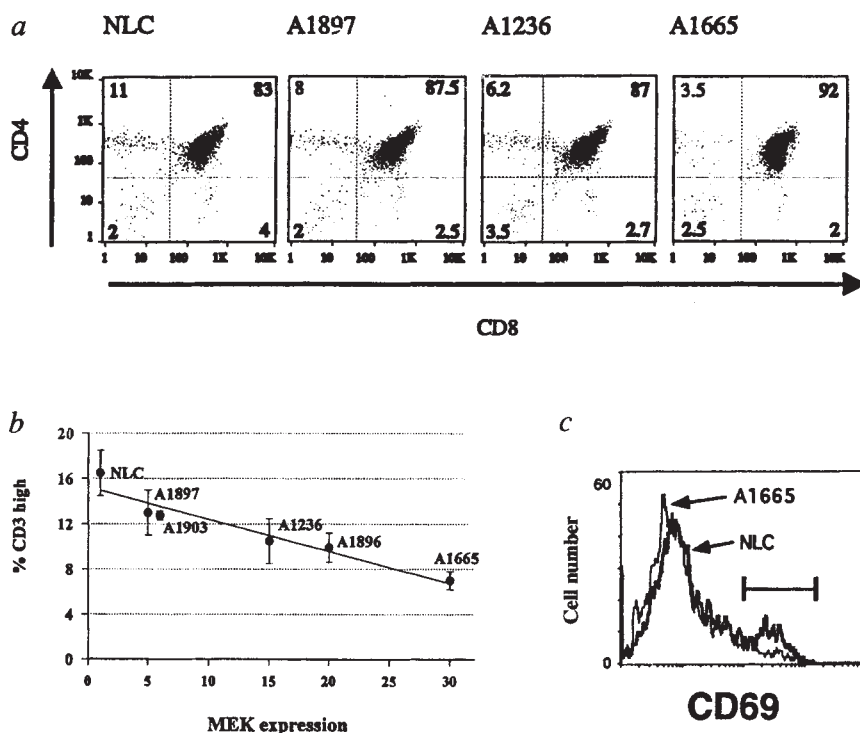
Despite apparent inhibition of the MAPK pathway, the thymuses in *lck*-MEK-1_{A97} transgenic mice displayed normal cellularity. Hence the proliferative steps associated with early thymocyte maturation¹³ were not grossly perturbed by marked inhibition of MAPK signalling. However, flow cytometric analysis of thymocytes from *lck*-MEK-1_{A97} transgenic mice revealed a substantial reduction in the numbers of mature CD4⁺8⁺ and CD4⁺8⁻ (single-positive) cells, of the type that normally express high levels of the TCR/CD3 complex, which correlated directly with the amount of defective MEK-1 protein expressed (Fig. 2a, b). This effect required expression of the catalytically inactive MEK-1 protein, because mice expressing fivefold-increased levels of wild-type MEK-1 under the control of the same promoter had normal thymuses (data not shown). Expression of *lck*-MEK-1_{A97} also produced a profound, dose-dependent decrease in the proportion of thymocytes expressing high surface levels of CD69 (Fig. 2c). This population primarily includes cells that have received a TCR-derived signal directing their maturation from the CD4⁺8⁺ double-positive to the single-positive stage¹⁴. Together these results suggest that activation of the MAPK cascade is required for maturation of single-positive thymocytes.

It should be noted that the generation of mature single-positive thymocytes still proceeds to some extent even in A1665 mice. The representation of these more mature cells is inversely

and linearly related to the level of expression of MEK-1_{A97} protein (Fig. 2b), suggesting that an even more profound disruption would be produced if a higher level of transgene expression could be achieved. In addition, this linear dose-response relationship suggests that the MAPK-influenced maturation of single-positive cells does not involve cell replication, an interpretation consistent with the known partitioning of replicating cells during thymopoiesis¹³.

To facilitate the examination of MAPK involvement in the maturation of single-positive thymocytes, we crossed mice from the A1665 and A1236 lines to mice bearing a transgene encoding a TCR specific for a male (H-Y) antigen in the context of the H-2D^b class I molecule. This model system has been used for the analysis of both positive and negative selection events: in adult male animals, virtually all thymocytes are deleted during development through interaction of the H-Y-specific TCR with its cognate ligand, whereas in female mice, selection of CD4⁺8⁺ class I-restricted thymocytes proceeds very efficiently^{15,16}. Analysis of the HY/*lck*-MEK-1_{A97} double-transgenic animals showed no difference in the extent of thymocyte deletion in males, indicating that negative selection is not affected by the presence of catalytically inactive MEK-1 (Fig. 3a). Activation-induced apoptosis, analysed *in vitro*, was similarly unaffected (data not shown). In contrast, the generation of CD4⁺8⁺ thymocytes in female mice bearing the TCR $\alpha\beta$ transgene was dramatically reduced in a dose-dependent manner by the presence of the *lck*-MEK-1_{A97} transgene (Fig. 3b). This effect is best visualized by gating on cells undergoing positive selection, which express high levels of the transgene-encoded TCR α -chain¹⁴. Here a 95% reduction in the proportion of CD4⁺8⁺ cells is typically observed in the HY/A1665 crosses (Fig. 3b). Hence overexpression of catalytically inactive MEK-1 blocks antigen-specific positive selection almost completely, while leaving negative selection intact. Interestingly, the generation of CD4⁺8⁻ thymocytes expressing high levels of the transgenic α -chain (Fig.

FIG. 2 Perturbation of thymocyte development in *lck*-dMEK mice. **a**, Thymocytes from 6-week-old mice were stained with anti-CD4-FITC and anti-CD8-PE and analysed by flow cytometry. Shown are two-colour histograms, indicating the percentage of cells in each quadrant. The total number of thymocytes present in each animal was similar. **b**, The percentage of cells expressing high levels of the TCR/CD3 complex (CD3^{hi} cells) in the different animals was determined by staining with anti-CD3 ϵ -FITC. The results of this analysis are plotted as a function of the levels of expression of the *lck*-MEK-1_{A97} transgene. Bars denote one standard deviation for $n \geq 3$ in each case, except A1903 where only two animals were analysed and the range of values is shown. **c**, The representation of thymocytes expressing high levels of CD69, a marker for cells that have received a positively selecting signal¹⁴, is shown for an A1665 *lck*-MEK-1_{A97} transgenic mouse (thin line) and a normal littermate control (NLC; thick line). **METHODS**. Flow-cytometric analysis was done essentially as described¹². Thymocytes were stained with saturating concentrations of antibody at 4 °C for 30 min. Cells were examined for surface expression of the following molecules: CD4 (PE L3T4, CalTag), CD8 (FITC Ly-2, CalTag), CD3 ϵ (FITC-conjugated 145-2C11) and CD69 (biotinylated H1.2FC; Pharmingen). Biotinylated antibodies were visualized using phycoerythrin- or trichrome-labelled streptavidin (CalTag). Analyses were done using a FAC-Scan flow cytometer (Becton-Dickinson) under Lysis II or Consort 30 software. Subsequent data analysis used Reproman software (True Facts Software, Seattle, WA).



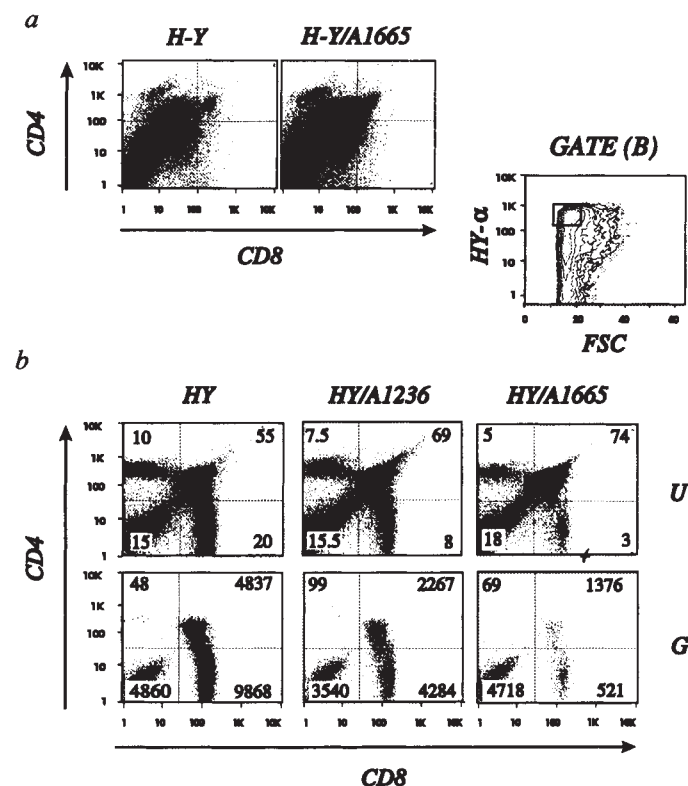
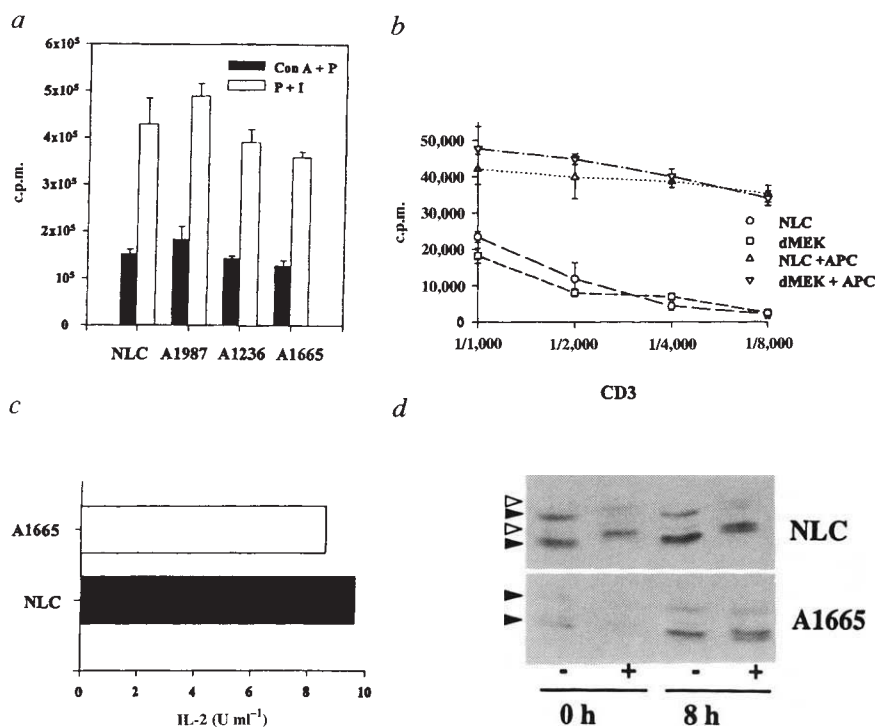


FIG. 3 Effect of MEK-1_{A97} expression on positive and negative selection of transgenic thymocytes bearing H-Y $\alpha\beta$ TCR. Thymocytes from male or female F₁ progeny of H-Y $\alpha\beta \times$ *lck*-MEK-1_{A97} (A1665 or A1236 lines) crosses were analysed for expression of CD4 and CD8. **a**, In males, the staining profiles of thymocytes from H-Y $\alpha\beta$ /*lck*-MEK-1_{A97} double-transgenic animals are indistinguishable from those of single-transgenic H-Y $\alpha\beta$ littermates, demonstrating that negative selection still occurs. **b**, In females, the generation of CD4⁺CD8⁺ cells in the double transgenic thymuses is markedly reduced. This is apparent in populations of total thymocytes, and becomes more evident when cells that express high levels of the α -chain of the transgenic TCR are selectively counted using the criteria (GATE B) shown at the top right. Percentages of cells are indicated in each quadrant in the ungated populations; in the gated histograms the actual numbers of events (of 100,000 total) are displayed.

METHODS. Thymocytes were prepared and stained with anti-CD4-PE, anti-CD8-FITC and H-Y-specific anti-TCR α -chain (biotinylated T3.70; ref. 15) as previously described. Analyses are described in Fig. 2 legend.

FIG. 4 Normal proliferative responses in *lck*-dMEK thymocytes and splenic T cells. **a**, Total thymocytes from normal littermate controls (NLC) or transgenic animals were isolated and stimulated *in vitro* with either combinations of pharmacological agents (concanavalin A + PMA or PMA + ionomycin) or mAbs against CD3 crosslinked with plate-bound rat-anti-hamster Ig in the presence or absence of fixed antigen-presenting cells (APC). The proliferation of thymocytes from the transgenic animals was not significantly different from that of thymocytes from normal littermate controls, even when, as shown in **b**, stimuli were titrated to suboptimal doses. **c**, **d**, IL-2 release and MAPK activation. **c**, Amount of IL-2 released per ml of medium after 8 h stimulation with anti-CD3 ϵ mAb crosslinked with plate bound rat-anti-hamster Ig for splenic T cells from an A1665 animal versus that released by identically treated normal littermate control (NLC) cells. **d**, Immunoblot using anti-MAPK antiserum. Aliquots of the splenic T cells used to measure IL-2 release were treated with PMA (100 ng ml⁻¹) for 10 min, after which lysates were prepared. Subsequent SDS-PAGE fractionation and immunoblotting revealed ERK-1 and ERK-2 proteins. PMA treatment ordinarily stimulates MEK-1, resulting in phosphorylation of the ERK proteins that can be appreciated as a decrease in electrophoretic mobility ($\blacktriangleright$). The samples derived from A1665 thymocytes fail to show this mobility shift ($\blacktriangle$).

METHODS. **a**, **b**, Thymocyte proliferation. 3×10^5 viable thymocytes were plated in 200 μ l medium (RPMI 1640, 10% FCS (Hyclone), 2 mM glutamine, 100 U ml⁻¹ penicillin and 100 mg ml⁻¹ streptomycin). Cells were stimulated with the indicated combinations of mitogens at the following concentrations: 2 ng ml⁻¹ PMA (Sigma), 0.5 mg ml⁻¹ concanavalin A (Vector), 500 ng ml⁻¹ ionomycin (Calbiochem). No single treatment induced proliferation in thymocytes. For the assays with CD3, plates were coated with rat-anti-hamster Ig, and 145.2C11 ascites



(anti-CD3 ϵ) was then added at the indicated dilutions. APCs (10^4 per plate), obtained from peritoneal exudates and irradiated, were included in half the samples. Culture was for 72 h, after which cells were labelled with 1 μ Ci ³H-thymidine (Amersham) for 6–8 h. ³H incorporation was determined as described²⁹ and normalized to the numbers of single-positive cells present in the culture, as determined by flow cytometry. **c**, IL-2 production was measured in a biological assay with HT-2 cells. **d**, MAPK gel-shift assay, performed as described⁸.

3b), cells that represent a distinct maturation pathway¹⁷, was not compromised.

Although the importance of the MAPK cascade has been emphasized with respect to the regulation of proliferative responses to growth-factor-derived signals, we have found that intrathymic proliferative events, confined largely to immature cells, proceed unimpaired in *lck*-MEK-1_{A97} mice. Moreover, those single-positive thymocytes that successfully developed in *lck*-MEK-1_{A97} mice proliferated normally following mitogenic stimulation. This was true irrespective of whether combinations of pharmacological agents (such as concanavalin A plus phorbol esters; Fig. 4a), immobilized anti-CD3 (Fig. 4b), or allogeneic cells (data not shown) provided the activating signal. Titration of the anti-CD3 response did not reveal even subtle inhibition of the proliferative response of *lck*-MEK-1_{A97} thymocytes (Fig. 4b). This normal mitogenic response evolved in the absence of appreciable MAPK activation, and did not result from inactivation of the transgene or elimination of the MEK-1_{A97} product. Indeed, stimulation of A1665 splenic T cells, in which the abundance of MEK-1_{A97} protein exceeds endogenous MEK-1 levels by only 10-fold, provoked normal release of interleukin-2 (IL-2) eight hours after stimulation (Fig. 4c), even though activation of MAPK, judged in this case by the ability of phorbol esters to provoke a characteristic alteration in MAPK electrophoretic mobility, was not observed (Fig. 4d).

Proliferative responses to TCR stimulation are blocked by expression of dominant-negative *ras*^{18,19}, but not by dominant-negative MEK-1 (Fig. 4). The signal-transduction pathway leading from the TCR complex may bifurcate at the level of p21^{ras}, permitting activation of MAPK-independent signalling elements which regulate T-cell replication. The JNK1 kinase system might well subserve this function²⁰. Irrespective of the precise mechanisms involved, it is apparent that positive selection is very sensitive to inhibition of the MAPK cascade, whereas both negative selection and proliferation proceed unimpaired even when no MAPK activation is detectable.

We note that studies in invertebrates also implicate MAPK in the control of differentiation. In *Drosophila*, maturation of R7 photoreceptor cells from immature progenitors requires signalling from the *Sev*-encoded protein tyrosine kinase, which acts through MEK-1 and MAPK analogues to augment the activity of a transcription factor, PntP2, at the expense of a second trans-acting factor (Yan) which ordinarily behaves as a repressor^{10,21,22}. Similarly, MAPK activation provides crucial signals governing the satisfactory differentiation of vulval cells in *C. elegans* from uncommitted progenitors¹¹. Our studies suggest that an analogous process regulates thymocyte development, beginning with the activation of protein tyrosine kinases known to couple to the TCR complex (p56^{lck}, p70^{ZAP})²³, followed by accumulation of p21^{ras} in its GTP-bound form²⁴, which recruits the kinase Raf-1 to the membrane, where it is activated²⁵, thereby initiating the MAPK cascade. Our results show that efficient differentiation of mature T lymphocytes required MAPK activation at a single, pivotal developmental stage. Thus activation of MAPK by signals from protein-tyrosine-kinase-associated surface receptors regulates lineage commitment in three evolutionary distant systems. □

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Axl receptor tyrosine kinase stimulated by the vitamin K-dependent protein encoded by growth-arrest-specific gene 6

Brian C. Varnum*, Cynthia Young*, Gary Elliott*, Andy Garcia*, Timothy D. Bartley*, Yih-Wol Fridell†, Robert W. Hunt*, Geraldine Trail*, Chris Clogston*, Robert J. Toso*, Donna Yanagihara*, Larry Bennett*, Maura Sylber*, Lee Anne Merewether*, Alice Tseng*, Eva Escobar*, Edison T. Liu† & Harvey K. Yamane*

*Amgen Inc., Amgen Center, Thousand Oaks, California 91320-1789, USA

†Department of Medicine, Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 2759-7295, USA

THE Axl receptor tyrosine kinase was identified as a protein encoded by a transforming gene from primary human myeloid leukaemia cells by DNA-mediated transformation of NIH 3T3 cells^{1–3}. Axl is the founding member of a family of related receptors that includes *Eyk*⁴, encoded by a chicken proto-oncogene originally described as a retroviral transforming gene, and *c-Mer*⁵, encoded by a human proto-oncogene expressed in neoplastic B- and T-cell lines. The transforming activity of Axl demonstrates that the receptor can drive cellular proliferation. The function of Axl in non-transformed cells and tissues is unknown, but may involve the stimulation of cell proliferation in response to an appropriate signal, namely a ligand that activates the receptor. We report here the purification of an Axl stimulatory factor, and its identification as the product of growth-arrest-specific gene 6 (ref. 6). This is, to our knowledge, the first description of a ligand for the Axl family of receptors.

Axl-expressing tumour cell lines were used as target cells for the development of a receptor autophosphorylation assay. This assay was used to screen conditioned media from 70 cell lines for a factor that stimulates the kinase activity of Axl. Two cell lines, Wi38 and Hs27, were found to produce an activity that greatly elevated the phosphotyrosine content of Axl (Fig. 1a), as measured by probing western blots of Axl immunoprecipitations with anti-phosphotyrosine antibodies. Furthermore, addition of

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Axl extracellular domain (Axl-x) at $1 \mu\text{g ml}^{-1}$ or 100 ng ml^{-1} blocked the phosphorylation response (shown for Wi38 conditioned medium (CM); Fig. 1b). The ability of Axl-x to block the phosphorylation response suggested that the stimulatory activity interacts with Axl directly, and was not stimulating phosphorylation by an indirect mechanism. Axl stimulatory activity in Wi38 and Hs27 CM was confirmed by an assay which measures receptor downregulation as an indication of ligand binding (G.E., manuscript in preparation). Exposure of A172 cells to Wi38 CM for 1 h resulted in a reduction of surface staining by 60–70%. Axl-x blocked the downregulation response as well. Axl downregulation was also observed in pulse-chase experiments with metabolically labelled protein, demonstrating that decreased surface staining was not due to epitope masking (data not shown).

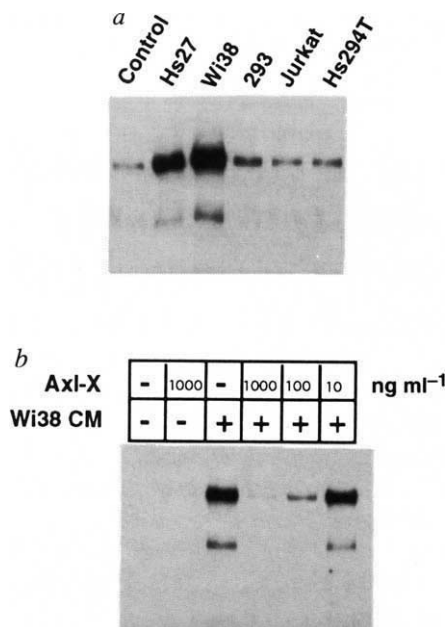


FIG. 1 Identification of an Axl stimulatory factor in cell culture supernatants. **a**, Medium conditioned by the indicated cell lines was screened for the ability to elevate the phosphotyrosine content of Axl on A172 cells. Lysates of treated cells were immunoprecipitated with anti-Axl antibodies¹⁴, resolved by SDS-PAGE, electroblotted, and probed with anti-phosphotyrosine antibodies (4G10, UBI). **b**, Competition of the phosphorylation response with soluble Axl extracellular domain (Axl-x). Axl-x was added to Wi38 conditioned medium and then tested for the ability to block the phosphorylation response.

METHODS. Conditioned medium was generated from cells at high density in the appropriate growth media at low serum (0.5%) for 6 days. A172 cells were plated in 6-well plates at 5×10^5 cells per well. After 24 h, the growth medium was removed and the test medium applied for 20 min. Cells were then lysed in PBS, 1% Nonidet P-40. Lysates were clarified by centrifugation and immunoprecipitated with polyclonal affinity-purified anti-Axl antibodies. Immune complexes were recovered with Protein-A agarose beads (Oncogene Sciences), washed with lysis buffer, solubilized in SDS-PAGE sample buffer, resolved on 8% gels, transferred to Immobilon-P (Millipore) and probed with anti-phosphotyrosine antibody. The blots were developed using chemiluminescence (ECL, Amersham). To produce Axl-x, a cDNA fragment encoding the extracellular domain of Axl with six histidines at the C terminus was cloned into pVL1392 (Invitrogen). Axl-x was produced in Hi-5 cells infected with a recombinant virus. In order to purify Axl-x, Hi5 conditioned media was concentrated using a S1Y30 spiral membrane cartridge and diafiltered against a buffer of 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, pH 7.9. The concentrate was applied to a 5 ml HiTrap Chelating Sepharose column (Pharmacia) equilibrated in the same buffer, washed with steps of 20 and 40 mM imidazole in binding buffer, and eluted with 100 mM imidazole in binding buffer. Fractions containing highly purified Axl-x were pooled, concentrated, and further purified on a 2.6×60 cm Superdex 200 column in 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, pH 7.9.

The phosphorylation and downregulation assays indicated that Wi38 CM contained a factor with activities expected of a ligand for a receptor tyrosine kinase.

The Axl stimulatory factor was purified from 60 l of Wi38 CM by successive chromatographies on Q-Sepharose Fast Flow (Pharmacia), hydroxylapatite (Bio-Rad) and an Axl-x affinity matrix. This scheme resulted in the enrichment of a protein of relative molecular mass 75K (Fig. 2). Amino-terminal sequence analysis of electroblotted⁷ p75 revealed a single sequence, AFQVEEAKQGHLE (single-letter amino-acid code). A computer-based homology search⁸ resulted in the unambiguous assignment of this sequence to the protein encoded by the growth-arrest-specific gene 6 (Gas6), a protein with significant homology with protein S (ref. 9), a vitamin-K-dependent protein involved in anticoagulation processes. Sequence of an additional 302 amino acids derived from proteolytic cleavage of p75 confirmed this assignment. Antibodies generated against the N terminus of Gas6 were used to demonstrate that Hs27 CM also contains Gas6 protein (data not shown).

Purified recombinant human Gas6 (rhGas6), produced in Chinese hamster ovary (CHO) cells (Fig. 2, lane 5), stimulated detectable Axl phosphorylation at concentrations as low as 3 ng ml^{-1} with a maximal response obtained with 100 ng ml^{-1} rhGas6 (Fig. 3a). Downregulation of Axl was similarly maximal at 100 ng ml^{-1} , and readily detectable at 3 ng ml^{-1} (12%) (Fig. 3b). Protein S had no activity in either assay (data not shown). The specific activity of rhGas6, as measured by downregulation, increased roughly 50% when CHO cell medium was supplemented with vitamin K, a cofactor for the enzyme responsible for carboxylation of glutamic acid residues¹⁰. In addition, reactivity to an antibody directed against an N-terminal peptide of Gas6 decreased (Fig. 3c). Four of seventeen amino acids within this peptide are potential carboxylation sites; therefore, decreased antibody binding may be an indirect measure of car-

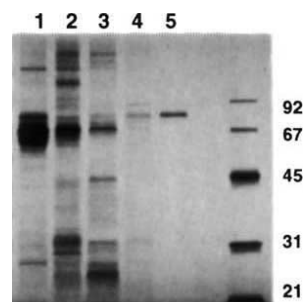


FIG. 2 Purification of the Axl stimulatory factor. Lane 1, Wi38 conditioned medium (10 μg total protein); lane 2, Q-Sepharose Fast Flow pooled fractions (10 μg); lane 3, Bio-Gel hydroxylapatite pooled fractions (5 μg); lane 4, Axl-x affinity column pooled fractions (2.5 μg); lane 5, purified rhGas6 (1 μg).

METHODS. Sixty litres of Wi38 conditioned media were concentrated using a S10Y10 spiral membrane cartridge and diafiltered against 10 mM Tris-HCl, pH 8.5. The concentrate was loaded on a 1.7 l Q-Sepharose Fast Flow (Pharmacia) column and eluted with a linear gradient of 0–0.5 M NaCl in 10 mM Tris-HCl, pH 8.5. Fractions containing peak Axl activity were pooled, concentrated, and diafiltered against 10 mM sodium phosphate, pH 7.2. The sample was then applied to a 15 ml Bio-Gel HTP (Bio-Rad) column and eluted with a gradient of 10–400 mM sodium phosphate buffer. Fractions containing peak activity were pooled, concentrated, and diafiltered against 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, pH 7.9. This material was applied to a 1 ml HiTrap Chelating Sepharose column (Pharmacia) charged with NiCl_2 and equilibrated with the same buffer, to which 200 μg of Axl-x had been applied. Bound material was eluted with a step of 4 M urea in the equilibration buffer. Following electroblotting onto PVDF, the major band at 75K was excised, subjected to gas-phase sequencing, and identified as the Gas6 gene product.

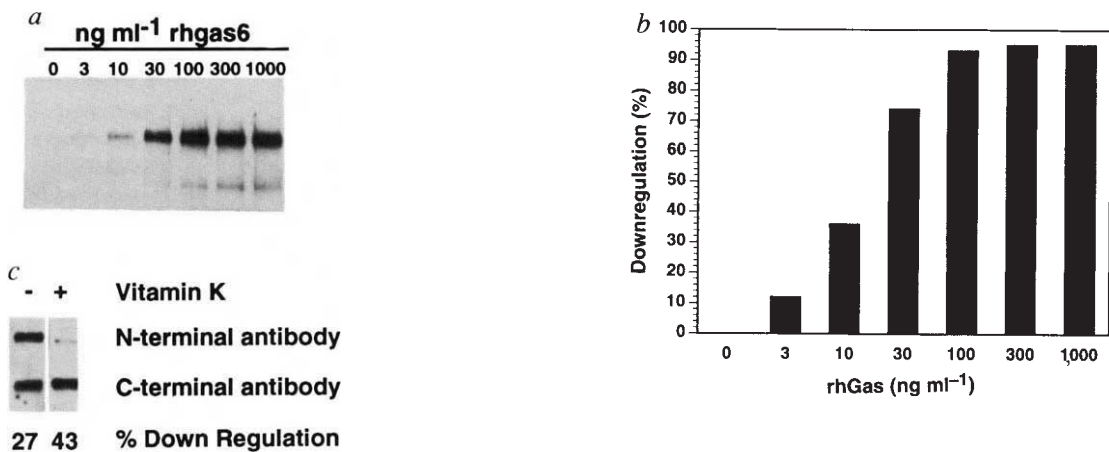


FIG. 3 a, Stimulation of Axl phosphorylation in A172 by purified rhGas6. b, Downregulation of Axl on A172 cells in response to rhGas6. c, Enhanced activity of rhGas6 produced in the presence of vitamin K. METHODS. A172 cells were treated with the indicated concentrations of rhGas6 for 20 min. The phosphotyrosine content of Axl was assessed as described in Fig. 1. Downregulation was determined by plating 2×10^5 A172 cells per well in a 96-well plate and incubating with 100 μ l of either control medium (DMEM) or DMEM with the indicated concentrations of rhGas6 for 1 h at 37 °C. Cells were then washed and stained for 45 min on ice with a phycoerythrin-conjugated anti-Axl monoclonal antibody. Cells were washed again and data acquired on a Becton-

Dickinson FACScan flow cytometer. Histograms and mean fluorescence values were analysed with LYSYS II software (B-D). rhGas6 was produced in CHO cells as follows. Human Gas6 cDNA was cloned into the mammalian expression vector pDSR-2, and CHO cells were transfected with this construct by calcium phosphate precipitation as described¹⁵. Vitamin K ($5 \mu\text{g ml}^{-1}$) was added to CHO cells expressing rhGas6. Protein levels were determined by blotting conditioned media with anti-Gas6 antibodies provided by C. Schneider. The N-terminal antibodies were raised against the N-terminal 17 amino acids of Gas6: AFQVFEEAKQGHLEEC. Activity was measured in a downregulation assay.

boxylation. These data suggest that vitamin-K-dependent carboxylation is required for full activity.

Binding of rhGas6 to Axl was demonstrated by chemical crosslinking of ^{125}I -labelled rhGas6 (1 nM) to A172 cells, followed by immunoprecipitation with anti-Axl antibodies. ^{125}I -rhGas6 was recovered in Axl immunoprecipitates (Fig. 4, lanes 1 and 2), and exposure to a crosslinker results in the appearance of a high-molecular-mass complex (lane 2). Unlabelled rhGas6 (20-fold molar excess) competed for ^{125}I -rhGas6 binding (lane 3). ^{125}I -rhGas6 was not detected in immunoprecipitations with anti-EGF receptor antibodies (lane 4). Demonstration of crosslinking at 1 nM, and phosphorylation and downregulation at subnanomolar concentrations, indicated that rhGas6 stimulates Axl at concentrations expected for an authentic ligand. rhGas6 also bound to purified Axl-x covalently coupled to a BIAcore sensorchip^{11,12}, with a dissociation constant of 4 nM.

Our results indicate that a vitamin-K-dependent protein known previously as a growth-arrest-specific gene is a ligand for Axl, an oncogenic receptor tyrosine kinase. This is the first identification of a ligand for a receptor of the Axl family, and may aid the search for ligands for other Axl-related receptors. The initial identification of Gas6 as a protein expressed in response to growth arrest suggested that it may function as a negative regulator of cell proliferation; however, the transforming ability of Axl suggested that activation of the receptor would stimulate proliferation. We have observed a 50% increase in 3T3 cell number after 7 days of treatment with 100 ng ml⁻¹ rhGas6 in 0.5% serum, but have failed to observe effects on A172 and Wi38 cell growth. Gas6 may stimulate DNA synthesis in serum-starved, but not density-arrested 3T3 cells, and this restricted mitogenic activity may be mediated by Axl (S. Goruppi and C. Schneider, manuscript in preparation). The potential role of Gas6 and Axl in cell proliferation is being further explored. The high degree of homology between Gas6 and protein S may provide additional clues to the function of this receptor-ligand system. It had been hypothesized that Gas6 may be part of a protease cascade involved in growth regulation⁶; however, our data now prove that, at least in part,

the activity of Gas6 is mediated by Axl. Additionally, protein S has been reported to be a mitogen for smooth muscle cells¹³. We suggest that this activity may involve activation of an Axl-related receptor. Demonstrating that Gas6 can function as a classical ligand for a receptor tyrosine kinase highlights a previously unrecognized potential activity for vitamin-K-dependent proteins. Isolation of additional ligands for Axl-related receptors will reveal whether additional γ -carboxylated proteins function as receptor ligands. The identification of Gas6 as a ligand for

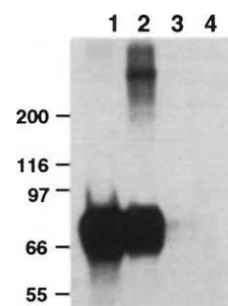


FIG. 4 Chemical crosslinking of ^{125}I -rhGas6 to Axl on A172 cells. Lane 1, Axl immunoprecipitation of A172 cells exposed to ^{125}I -rhGas6, 75 ng ml⁻¹; lane 2, as lane 1 with addition of crosslinker; lane 3, as lane 2 with addition of 1.5 $\mu\text{g ml}^{-1}$ unlabelled Gas6; lane 4, ^{125}I -rhGas6 + crosslinker, immunoprecipitated with anti-EGF receptor antibody (LA1, UBI).

METHODS. 5×10^5 A172 cells were plated in 100-mm dishes overnight. The cells were washed twice with PBS, and then placed on ice. Ice-cold PBS (4 ml) was added. Unlabelled rhGas6 ($1.5 \mu\text{g ml}^{-1}$) was added to one plate, then ^{125}I -rhGas6 (75 ng ml⁻¹) was added to each plate and binding allowed to proceed for 1 h on ice. Crosslinking was initiated with addition of 1 mM bis(sulphosuccinimidyl) suberate (Pierce), and continued for 30 min on ice. The cells were washed twice with TBS, then lysed and immunoprecipitated as described for Fig. 1. rhGas6 was iodinated by the chloramine-T method with a labelling kit from ICN to a specific activity of 7.5 c.p.m. μg^{-1} .

Axl will allow us to examine the role of this receptor–ligand system in the regulation of cellular processes. □

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SNAP-mediated protein–protein interactions essential for neurotransmitter release

W. M. DeBello^{*†‡}, V. O'Connor[§], T. Dresbach^{†§},
S. W. Whiteheart^{||}, S. S.-H. Wang^{*†},
F. E. Schweizer^{*†}, H. Betz^{†§}, J. E. Rothman^{||}
& G. J. Augustine^{*†}

^{*} Department of Neurobiology, Duke University Medical Center, Durham, North Carolina 27710, USA

[†] Marine Biological Laboratory, Woods Hole, Massachusetts 02543, USA

[§] Department of Neurochemistry, Max-Planck-Institute for Brain Research, 60528 Frankfurt, Germany
^{||} Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, New York 10021, USA

THE constitutive fusion of transport vesicles with intracellular membranes requires soluble proteins called SNAPs¹. Certain pre-synaptic proteins^{2–4} implicated in synaptic vesicle exocytosis⁵ also bind SNAPs, suggesting that SNAPs participate in the calcium-regulated membrane fusion events mediating neurotransmitter release^{6,7}. Here we show that injection of recombinant SNAPs into the giant synapse of squid enhances transmitter release. Conversely, injection of peptides designed to mimic the sites at which SNAP interacts with its binding partners inhibits transmitter release downstream of synaptic vesicle docking. A SNAP-dependent protein complex must therefore mediate transmitter release, showing that transmitter release shares a common molecular mechanism with constitutive membrane fusion.

We isolated a squid complementary DNA encoding a full-length SNAP (s-SNAP) with a predicted molecular mass of 33K. This squid SNAP (s-SNAP) is 67% identical to bovine α -SNAP (Fig. 1a) and 65% identical to bovine β -SNAP (not shown), but it is only 17% identical to γ -SNAP, a third, more distantly related mammalian isoform⁸, and 33% identical to Sec17, a yeast SNAP homologue (Fig. 1a). The homology between α/β -SNAPs across evolutionary divisions is reminiscent of other presynaptic proteins^{9,10} and suggests a conserved function for SNAPs in neurons.

To test whether SNAPs are involved in transmitter release, we injected recombinant s-SNAP into the cytoplasm of the giant presynaptic terminal while monitoring release evoked by single presynaptic action potentials¹¹. The rate of rise (dV/dt) of the postsynaptic potential response (p.s.p.) after SNAP injection was faster (Fig. 1b, c), showing that the protein enhanced transmitter release. The maximal enhancement ($14.3 \pm 3.3\%$ s.e.m.; $n = 15$) occurred during or shortly after s-SNAP injection and then declined slowly.

TABLE 1 Effects of microinjected proteins and peptides on synaptic transmission

Reagent	No. of injections (reversals)	Effect on release mean (s.e.m.)
Fusion proteins		
s-SNAP (His-6 fusion protein)	15 (15)	+14.3% (3.3)*
α -SNAP (His-6 fusion protein)	15 (15)	+23.2% (3.5)*
γ -SNAP (His-6 fusion protein)	7 (7)	+12.8% (2.1)*
Sec17 (His-6 fusion protein)	5 (n/a)	+1.4% (3.4)
Peptides		
SS19 (s-SNAP 19-mer)	42 (33)	–83.8% (2.9)*
scSS19 (scrambled SS19)	6 (n/a)	–9.2% (4.9)
ceSS19 (charge-exchanged SS19)	3 (3)	–52.7% (9.5)*
SS13 (SS19 N-terminal domain)	7 (7)	–71.6% (8.9)*
nscSS19 (SS19 with scrambled N-terminal domain)	7 (n/a)	–12.0% (3.8)
SS24 (s-SNAP 24-mer)	10 (2)	–71.6% (4.6)*
MS20 (α -SNAP 20-mer)	19 (0)	–77.5% (7.5)*
MS18 (α -SNAP 18-mer)	4 (n/a)	–7.8% (4.8)
Carrier (Peptide carrier solution)	10 (n/a)	–13.0% (5.5)

The amount of transmitter released in response to each stimulus was determined by measuring the slope of the p.s.p. at a potential level below action potential threshold. This value was measured during the peak effect of each injection and divided by the pre-injection value to yield the percentage change in release.

* The effect is significantly different from carrier solution controls ($P < 0.05$, Student's *t*-test).

This enhancement is consistent with the ability of SNAPs to promote intracellular vesicle fusion underlying Golgi transport¹². *In vitro*, Golgi vesicle fusion requires only two soluble proteins, SNAP¹² and NSF^{12,14} (*N*-ethylmaleimide-sensitive factor), an ATPase attached to membranes by SNAP^{12,15}. α -SNAP is necessary for Golgi transport, whereas γ -SNAP facilitates transport with lower efficiency than α -SNAP¹². Microinjection of either protein stimulated transmitter release, with α -SNAP being approximately twice as effective as γ -SNAP (Table 1). Yeast Sec17, which supports mammalian Golgi transport only weakly *in vitro* (S.W.W., unpublished results), had no effect on transmitter release (Fig. 1d and Table 1). The relative abilities of SNAP isoforms to stimulate transmitter release thus paralleled their capacity to support constitutive vesicle–membrane fusion *in vitro*¹², indicating strong similarities in the molecular mechanisms underlying each process.

SNAPs are involved in several protein–protein interactions thought to be essential for membrane fusion^{6,7,12,15}. To disrupt these, we synthesized peptides mimicking regions of s-SNAP and α -SNAP that could participate in such interactions (Fig. 1a). Some of these dramatically inhibited synaptic transmission on microinjection (Fig. 2; Table 1). Active peptides included a 19-amino-acid peptide from the N-terminal region of s-SNAP, termed SS19 (for squid SNAPtide 19-mer), SS24 from an adjacent N-terminal domain, and MS20 from a central domain in mammalian α -SNAP. MS18, from a central domain of α -SNAP which is 72% identical to s-SNAP, had no effect (Table 1). The effects of SS19 were rapidly and completely reversible (Fig. 2a, b). This reversal, presumably due to diffusion of peptide into

[‡] To whom correspondence should be addressed at Duke University.

^{||} Present address: Department of Biochemistry, University of Kentucky College of Medicine, Chandler Medical Center, 800 Rose Street, Lexington, KY 40536-0084, USA.

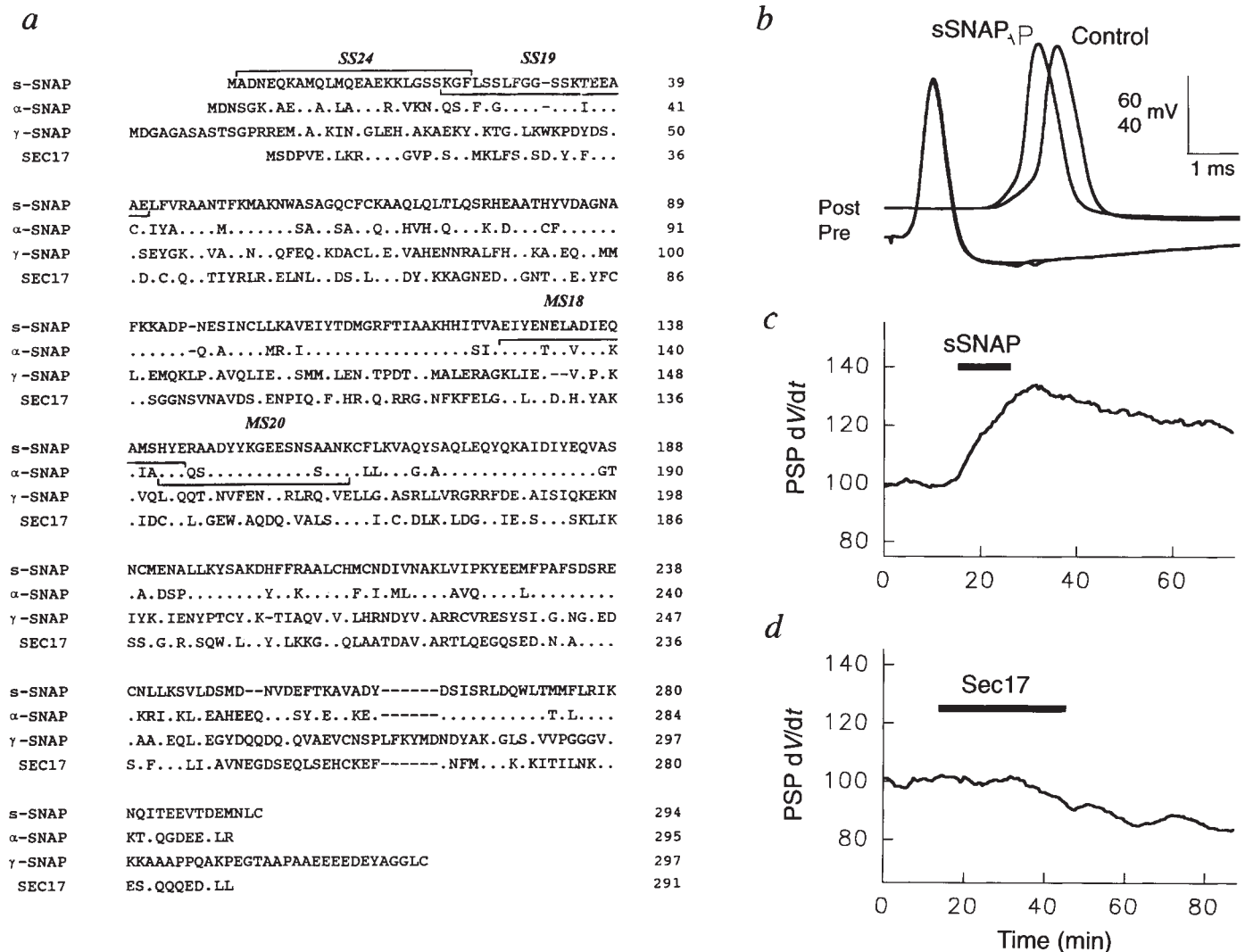


FIG. 1 a, Amino-acid sequence alignment of s-SNAP with bovine α -SNAP, γ -SNAP and yeast Sec17. Identical residues are indicated by dots; gaps introduced to optimize sequence identities are marked by dashes. Solid lines indicate the s-SNAP (SS) or mammalian α -SNAP (MS) sequences from which peptides were synthesized. These regions were selected on the basis of evolutionary conservation, predicted surface exposure and/or coiled-coil conformation, all common features of regions of protein-protein contact²⁹. **b**, Recordings of presynaptic (Pre) and postsynaptic (Post) potentials during microinjection of s-SNAP. **c**, Time course of enhancement of release produced by s-SNAP (injected during the bar). P.s.p. rate-of-rise (dV/dt) was normalized relative to its mean pre-injection value. **d**, Lack of effect of Sec17 injection. **METHODS**. A λ gt10 squid optic lobe cDNA library (2×10^6 PFU) kindly donated by J. Battey (NIH) was screened under low stringency (20% formamide, 43 °C) using a dioxygenin-modified rat β -SNAP probe; labeling, washing and immunological detection of positive hybridization were carried out as prescribed by the manufacturer of the dioxygenin-labeling system (Boehringer). The probe, validated by sequencing, was generated from rat brain cDNA by polymerase chain reaction (PCR; 30 cycles at 94 °C, 55 °C and 72 °C, for 60 s each) using sense and antisense oligonucleotides derived from the nucleotide sequences of bovine β -SNAP encoding amino acids 49–55 and 176–182, respectively. The 20 strongest hybridization-positive plaques were purified and used to identify 2 overlapping clones that contained the full-length open reading frame with an upstream Kozak translation initiation consensus

sequence. The cDNA sequence of s-SNAP has been submitted to the Genbank/EMBL data library under the accession number X82847. Electrical measurements and microinjections were performed as previously described⁹. Transmitter release was evoked every 30 s, by a current-passing microelectrode in the presynaptic axon. A second postsynaptic electrode was used to record the resultant p.s.p.; a third electrode in the presynaptic terminal was used for microinjection and potential recording. The microinjection carrier solution included 50 mM Na-HEPES, 200 mM KCl, 100 mM taurine, 200 mM K-isethionate, protein (0.2–1.0 mg ml⁻¹) and either fura-2 (0.5 μ M) or fluorescein-dextran (M_r 10K; 0.1 mM; both fluorescent indicators from Molecular Probes). N-terminally His-tagged fusion proteins encompassing the full-length sequences of bovine α - and γ -SNAPs and yeast Sec17 were prepared as described⁸. For s-SNAP, a full-length cDNA was constructed by PCR amplification of the 5' and 3' portions followed by ligation via a unique *Bsm*AI restriction site. The full-length cDNA was then subcloned into pQE9 for production of recombinant protein. A Deltascan photometer (PTI, New Jersey) was used to monitor fluorescence changes that occurred during protein microinjection. Solutions were microinjected by brief pulses of nitrogen gas delivered from a Picospritzer (General Valve, New Jersey). Physiological saline (13–16 °C) contained either normal (11 mM) or reduced (5.5 mM) Ca²⁺ concentration. Data were acquired by 'Tomahacq', a program written by T. Goldthorpe (University of Toronto) and analysed off-line by 'Squid', written by F.E.S.

the presynaptic axon^{9,11}, argues against the possibility that inhibition results from presynaptic damage. A control peptide (scSS19), containing the same amino acids as SS19 but in a scrambled order (Fig. 2c), had no effect (Fig. 2d; Table 1), confirming that the action of SS19 is sequence-specific.

To investigate the structural requirements for SS19 inhibition, we examined the actions of several SS19 analogues (Fig. 2c). The SS19 sequence can be divided into an N-terminal hydrophobic domain (amino acids 1–12 in Fig. 2c), and a C-terminal charged domain (amino acids 13–19). An SS19 analogue in which oppositely charged residues were exchanged, ceSS19, also inhibited transmitter release (Table 1), indicating that the charged residues are not essential for peptide action. Indeed, an analogue in which the charged residues were identical to those of SS19, but in which the order of N-terminal amino acids was scrambled (nscSS19), had no effect (Table 1), and a 13-mer representing the N-terminal domain alone (SS13) inhibited release reversibly (Fig. 2e). Thus, the N-terminal domain of SS19 plays a key role in SNAP-mediated protein–protein interactions that are essential for release.

The specificity of these peptide actions was characterized further by examining their effects on intercisternal Golgi transport. SS19 completely blocked transport *in vitro* (Fig. 3a), presumably by inhibiting the fusion of transport vesicles with the

Golgi membrane. All analogues of SS19 that inhibited transmitter release, such as ceSS19, SS13 and SS24 (not shown), also inhibited transport, whereas the inactive peptides scSS19, nscSS19 and MS18 had little or no effect at concentrations as high as 1 mM (Fig. 3a). SNAP-mediated protein–protein interactions are thus important for both membrane fusion events.

One critical step that could be affected by SS19 is the rise in presynaptic calcium ion concentration that triggers synaptic vesicle (SV) fusion^{16,17}. To test this, we used fura-2 to measure Ca^{2+} levels in presynaptic terminals^{18,19} stimulated by trains of presynaptic action potentials (Fig. 3b). The mean amplitude of stimulus-evoked changes in fura-2 fluorescence measured in the presence of SS19 was 90% (s.e.m. 5%) of those produced by identical stimuli before injection or after recovery. This slight decrease cannot account for the complete inhibition of neurotransmitter release by SS19.

To determine the kinetics of inhibition, we injected SS19 while stimulating the synapse rapidly (once every 2 seconds). A single injection produced a measurable decrease in transmitter release within two stimuli, showing that SS19 acts within 4 s (Fig. 3c). SS19 therefore acts at least as quickly as synaptotagmin peptides, which inhibit exocytosis downstream of synaptic vesicle docking⁹. By contrast, inhibition of earlier steps in synaptic vesicle trafficking by guanine nucleotide analogues requires tens of

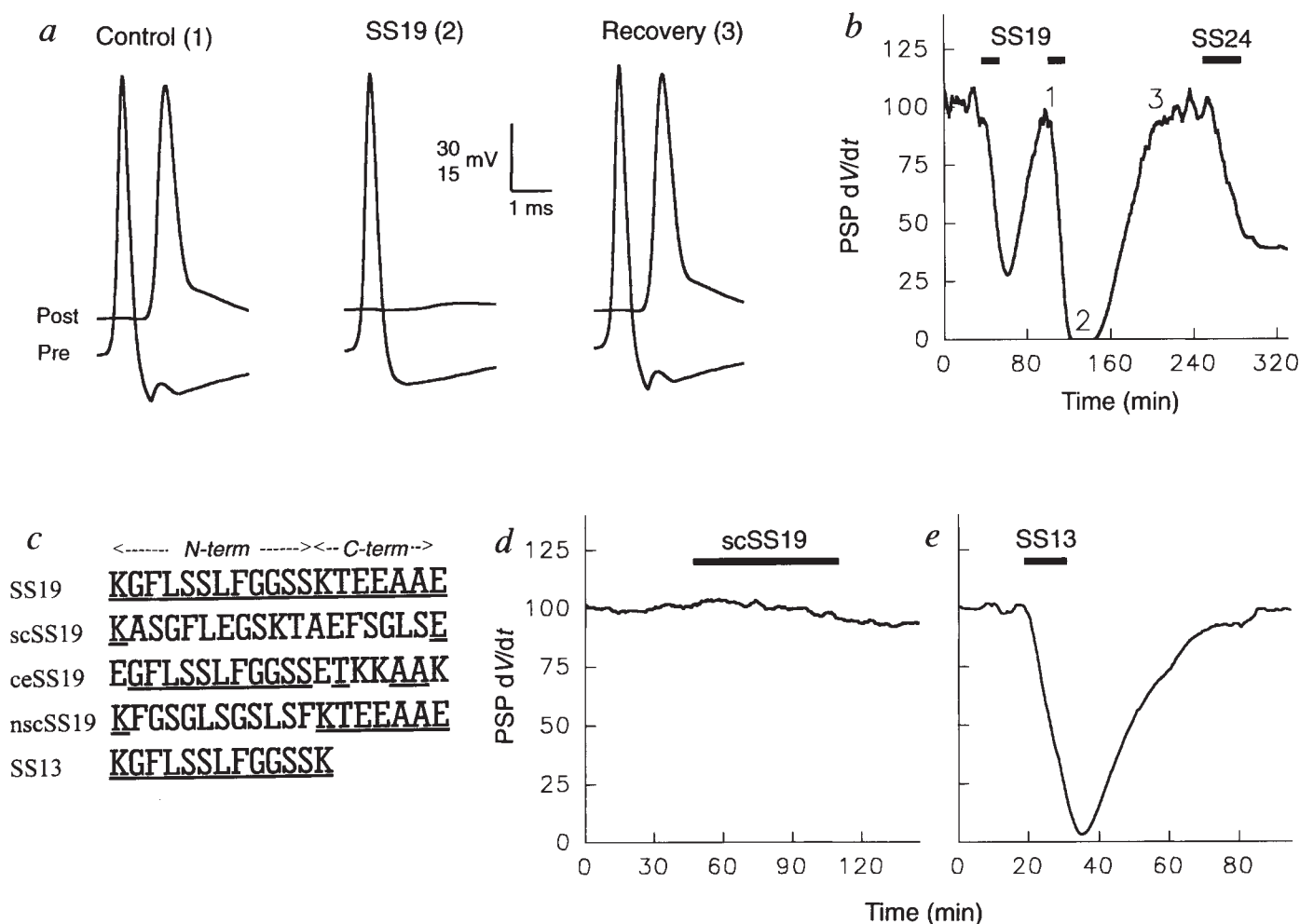


FIG. 2 s-SNAP peptides inhibit evoked transmitter release. **a**, Presynaptic (Pre) and postsynaptic (Post) potential recordings before, during and after microinjection of SS19. **b**, Time course of inhibition produced by SS19 or SS24 injection. Numbers indicate the times when the traces shown in **a** were acquired. **c**, Sequences of SS19-derived peptides. Residues identical to SS19 sequence are underlined. **d**, Microinjection

of scSS19 did not affect release, whereas SS13 (**e**) did inhibit reversibly. **METHODS.** All peptides, synthesized by Neosystems (Strasbourg, France), Chiron Mimotopes Peptide Systems (Sydney, Australia) or Bio-Synthesis, Inc. (Lewisville, TX) contained alkylated N-termini and amidated C-termini. Peptides were dissolved in carrier solution (100 mM taurine, 200 K-isethionate, 50 HEPES) at 2–30 mM (typically 20 mM).

minutes²⁰. Because a vesicle takes at least 30 s to complete a cycle of exo-endocytosis^{21,22}, SNAP must play a role in a step temporally close to exocytosis.

To investigate the stage of synaptic vesicle cycling that is regulated by SNAP, we used electron microscopy^{9,10,20} to visualize the distribution of synaptic vesicles in presynaptic terminals injected with SS19. SS19 dramatically altered the spatial distribution of SVs at active zones (Fig. 3*d, e*). The number of vesicles located within 50 nm of the active zone membrane increased almost

threefold (Fig. 3*f, g*). This subset of synaptic vesicles corresponds to those docked at the active zone^{9,10,20}, and is thought to include those participating in exocytosis²³. When release was inhibited by injection of SS19 and allowed to recover before fixation, the distribution of synaptic vesicles was similar to control terminals (Fig. 3*h*). The fact that SS19 reversibly increases the number of docked vesicles in active zones indicates that SNAP participates in a step between vesicle docking and fusion. SS19 also reduced the number of cytoplasmic synaptic vesicles

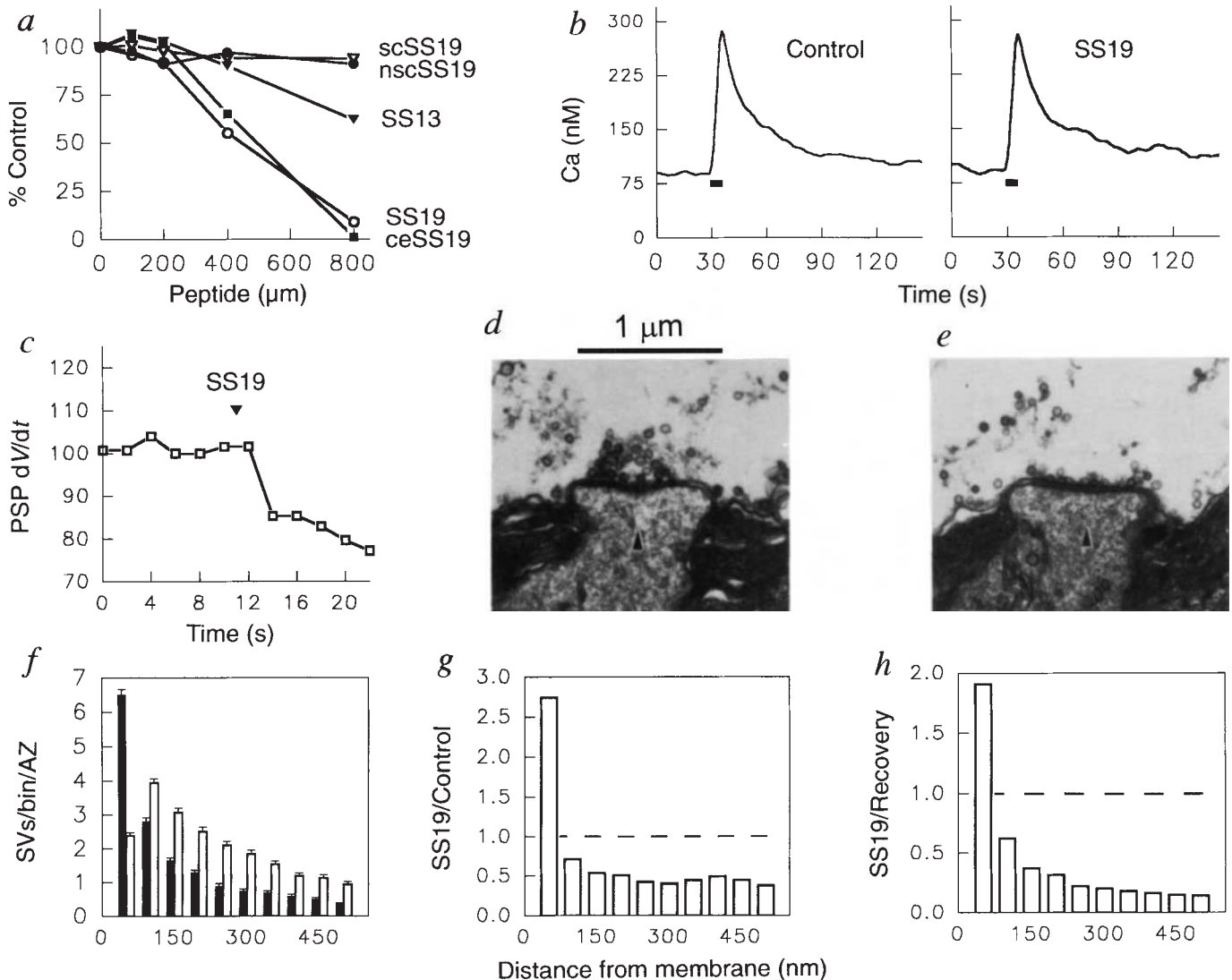


FIG. 3 Mechanism of action of SS19. *a*, Effects of SNAPtides on intra-Golgi transport. SS19 and ceSS19 inhibit transport with an ED_{50} of ~ 400 μ M; SS13 is less effective and scSS19 and nscSS19 have no effect even at 1 mM. *b*, Time course and amplitude of presynaptic Ca^{2+} rises measured by fura-2 were similar before (control) and after (SS19) injection of SS19. *c*, Rapid inhibition of release following a single large injection of SS19 (100 ms duration; arrowhead). *d* and *e*, Electron micrographs of active zones from terminals injected with inactive MS18 (*d*) or with SS19 and fixed at the peak of inhibition (*e*). Arrowheads are located in postsynaptic spines and point toward active zones. *f*, Distribution of the number of synaptic vesicles (SVs) per active zone (AZ) at various distances (50 nm bin size) from the presynaptic membrane of two terminals injected with SS19 (solid bars; $n=461$ AZs) and two control terminals (open bars; $n=391$ AZs). *g*, Relative densities of SVs in terminals injected with SS19 compared to ones injected with control solution, obtained by dividing data shown as solid bars in *f* by data shown as open bars. *h*, Relative densities of SVs in terminals injected

with SS19, as compared to one terminal injected with SS19 and then allowed to recover from inhibition ($n=231$ AZs).

METHODS. Golgi transport was measured in an *in vitro* transport assay³⁰ consisting of Golgi membranes from wild-type CHO cells (acceptor membranes) and membranes from VSV-infected 15B mutant CHO cells (donor membranes) together with CHO cell cytosol and an ATP-regenerating system. The reactions were incubated at 37 °C for 1 h and the glycosylated VSV-G protein was recovered by immunoprecipitation. Transport between compartments was measured as the incorporation of [3 H]-N-acetyl-glucosamine into the VSV-G protein transported from the glycosylation-deficient mutant Golgi membranes to the wild-type membranes (where glycosylation can occur). For electron microscopy, terminals were fixed, prepared and analysed as described^{9,10,20}. Sections of 80 nm thickness were acquired at 50- or 75- μ m intervals along the length of each presynaptic terminal and examined on a JOEL electron microscope. Active zone images were digitized and analysed using Image-1 software (Universal Imaging, Philadelphia).

at the active zone by 24% (Fig. 3f–h), indicating that it may also prevent replenishment of these vesicles following their translocation to membrane docking sites. SNAP may thus act at fusion steps in addition to that between the plasma membrane and synaptic vesicles²³.

These results constitute the first direct evidence that SNAPs, essential for constitutive vesicle-membrane fusion, also participate in Ca²⁺-regulated neurotransmitter release. Three presynaptic proteins—synaptotagmin⁹, synaptobrevin¹⁰ and SNAP—have now been implicated in steps downstream of docking. A SNAP-dependent protein complex therefore appears to perform a critical function after synaptic vesicles dock. This post-docking action requires binding to other proteins, as predicted by a model⁷ in which SNAP functions downstream of Ca²⁺ binding

to synaptotagmin²⁴. As this model postulates multiple binding and hydrolysis steps following calcium influx, the sub-millisecond interval between Ca²⁺ elevation and synaptic vesicle fusion^{11,25,26} may be insufficient time for these events to take place. SNAP may therefore prime the synaptic vesicle for exocytosis before synaptotagmin confers Ca²⁺-dependent regulation of fusion²⁷. Finally, our data show that SNAP concentration is rate-limiting for neurotransmitter release, implying that its regulation—by control of transcription or by post-translational modification—could have important physiological consequences. Because transmitter release is modulated during many different forms of synaptic plasticity²⁸, SNAP may be a target for the regulatory mechanisms underlying presynaptic plasticity. □

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Mitotic forces control a cell-cycle checkpoint

Xiaotong Li & R. Bruce Nicklas

Department of Zoology, Duke University, Box 91000, Durham, North Carolina 27708-1000, USA

EVERY time a cell divides, the chromosomes must be distributed accurately to the daughter cells. Errors in distribution arise if chromosomes are improperly attached to the mitotic spindle. Improper attachment is detected by a cell-cycle checkpoint in many cells^{1,2} and the completion of cell division is delayed, allowing time for error correction. How is an improperly attached chromosome detected? An absence of tension from mitotic forces is one possibility³. Here we test this possibility directly by applying tension to an improperly attached chromosome with a micromanipulation needle. In the absence of tension, the entry into anaphase and the completion of mitosis was delayed by 5–6 hours. When the misattached chromosome was placed under tension, however, the cell entered anaphase in 56 minutes, on average. Tension from mitotic forces or from a micromanipulator's needle evidently signals to the checkpoint that all is in order and that cell division can proceed.

The spermatocytes of praying mantids are prime examples of cells with a cell-cycle checkpoint sensitive to a single misguided chromosome. The spermatocytes normally contain a sex-chromosome trivalent formed from the pairing of a Y chromosome with two different X chromosomes, X₁ and X₂, (Fig. 1; 143-min image). Normal offspring will result only if the X₁ and X₂ segregate from the Y chromosome to produce sperm that contain

either both X chromosomes or the Y. This requires the attachment of the two X chromosomes to the same spindle pole and the Y chromosome to the opposite pole. The trivalent commonly attaches improperly at first (Fig. 1; 0 min), but that error is soon corrected (Fig. 1; 23–82 min)^{4,5}. Another type of error poses a different problem. In 10% of the cells, the three sex chromosomes fail to remain connected in a trivalent, and so they occur as an X-Y bivalent and a free X chromosome (Fig. 2; 0- and 0.1-min images). The free X chromosome segregates at random relative to the X-Y bivalent. Thus, as often as not, the daughter cells receive an inappropriate chromosome complement, as shown in Fig. 2 (69- and 69.1-min images), which would lead to sexually strange mantids in the next generation. The cell-cycle checkpoint prevents this. Cells with a free X chromosome are delayed in completing division and degenerate without forming sperm⁶. The cost of such damage control is a few sperm. This is a cheap price to pay as sperm are abundant compared with eggs and are wasted anyway if a male mates only once because his first partner makes a meal of him.

Studies of fixed material suggested that cells with a free X chromosome are arrested forever in metaphase⁶. Our examination of living cells discloses that the division of cells with a free X chromosome is not prevented altogether, but rather is delayed by 5 to 7 hours (sample of 15 cells). Normally, sperm develop synchronously. Perhaps the delayed cells are far enough out of synchrony that they cannot respond to the normal developmental signals and therefore fail to form sperm.

Somehow the checkpoint detects a single misguided chromosome. McIntosh³ suggested that the signal might be provided by spindle mechanics. He proposed that an absence of tension signals the checkpoint to delay division, whereas tension signals that all is well and that division can proceed (Fig. 3a). A properly

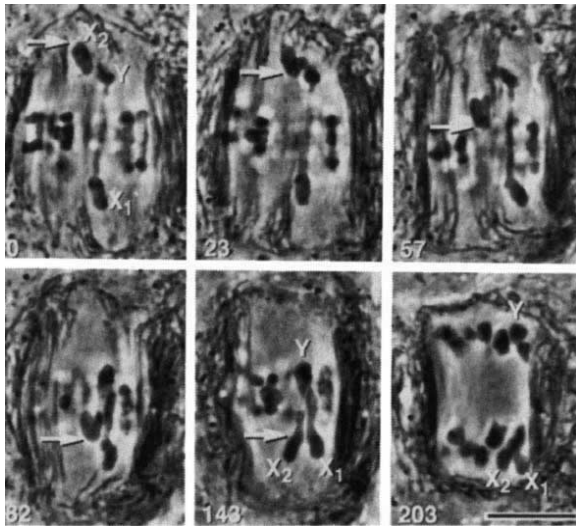


FIG. 1 Normal mantid spermatocyte division in a living cell, from prometaphase (0-min image) through to anaphase (203-min image). The time in minutes is given on each image. The sex chromosome trivalent attached to the spindle improperly at first (0-min image): one X chromosome (X_2) and the Y chromosome attached to the same spindle pole (the upper pole) while the other X (X_1) attached to the opposite (lower) pole. That error was spontaneously corrected by reorientation of the X_2 chromosome at the upper pole (23–82 min); it became attached to and moved towards the lower pole. As a result, the sex chromosomes segregated properly in anaphase (203-min image), the two X chromosomes separating from the Y chromosome. Scale bar, 10 μ m. Praying mantids, *Tenodera aridifolia sinensis* (Saussure), were reared from eggs supplied by Carolina Biological Supply Co. (Burlington, NC). The young mantids were fed *Drosophila*, then crickets and finally grasshoppers as they grew larger. Spermatocytes were cultured as described⁵. Chromosome behaviour was recorded on an optical disk recorder (model 2021, Panasonic Video Systems).

attached chromosome is subjected to forces towards opposite spindle poles that act at two attachment sites, the kinetochores (Fig. 3a). Hence, the two kinetochores and the chromatin between them are under tension. In contrast, an improperly attached chromosome has only one kinetochore connected to a

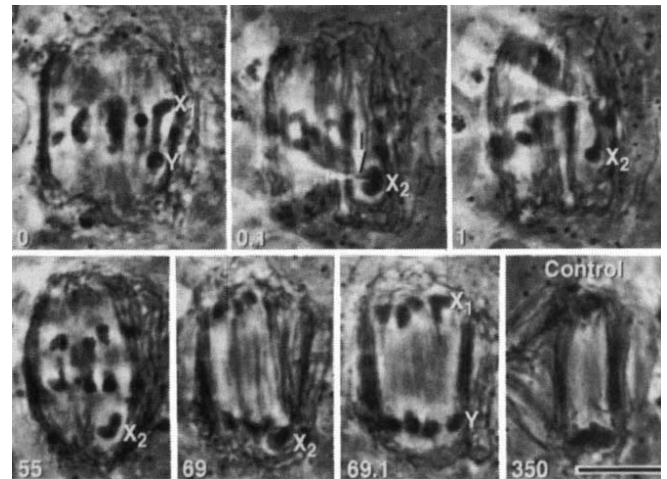
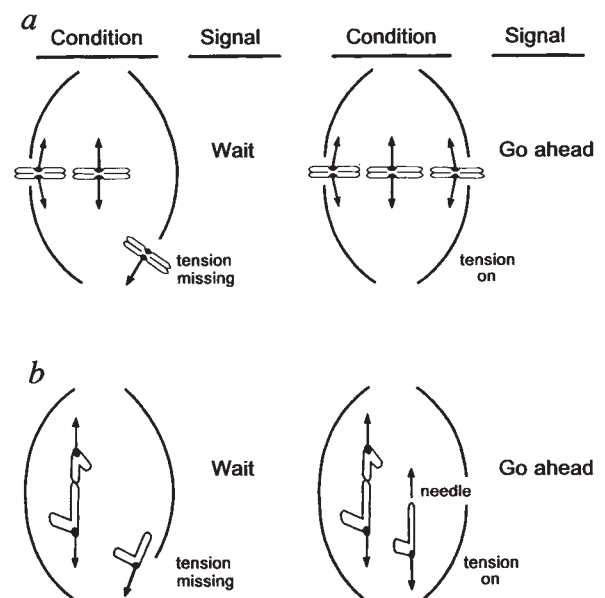


FIG. 2 A living mantid spermatocyte with an X-Y bivalent (X_1 , Y; 0-min image) and a free, unpaired X chromosome (X_2 ; 0.1-min image). The time in minutes is given on each image. The cell was arrested in metaphase owing to the presence of the unpaired X_2 chromosome. The X_2 chromosome was impaled with a micromanipulation needle (arrow, 0.1-min image), stretched (1 min), and thus held, with its kinetochore under tension. The cell entered anaphase in less than an hour (55–69.1 min); the sex chromosomes segregated properly. A cell nearby unmanipulated with an unpaired X chromosome entered anaphase 5 h later (control; 350-min image). Scale bar, 10 μ m. Spermatocytes were micro-manipulated by standard procedures⁵.

pole (Fig. 3a), and experiences force only towards that one spindle pole. Consequently, tension is absent. Rieder *et al.*¹¹ recently provided proof that a mammalian cell has a checkpoint sensitive to a single misattached chromosome. Like McIntosh, they speculated that it is the absence of tension that signals the checkpoint. We tested the proposal by micromanipulation of living cells. The experiment is simple in principle (Fig. 3b): pull on a free X chromosome in an arrested cell, placing its kinetochore under tension; does this lift the block to division? The cell in Fig. 2 had been arrested in metaphase for about 4 hours before the experiment began (so sister cells with normal sex-trivalents entered anaphase an average of 4 hours earlier). A

FIG. 3 Diagrams of checkpoint control by mechanical force. a, Mechanism proposed for cells in somatic mitosis³. Mitotic forces (arrows) act towards a spindle pole from the attachment sites, kinetochores, on each chromosome pair. Properly attached chromosomes are under tension from oppositely directed mitotic forces, but a chromosome attached to only one pole is not. The absence of tension signals the checkpoint to delay cell division. When the last chromosome becomes attached to both poles, and is under tension, this signals to the checkpoint that division can proceed. b, Experimental test in mantid cells in meiosis. An X-Y chromosome pair is under tension from mitotic forces, but a free unpaired X chromosome is not: division is delayed. When the missing tension is added with a micromanipulation needle, the cell goes on towards division.



microneedle was inserted in the free X chromosome and moved upwards, stretching the chromosome and applying tension to its kinetochore. After 55 min of tension, the cell entered anaphase (55–69.1 min images in Fig. 2). Control cells, cells with a free X chromosome not placed under tension, entered anaphase only after an additional 5 hours (350 min image in Fig. 2). The free X chromosome was subjected to tension in a total of 10 cells; these cells entered anaphase after an average of 56 min under tension (range, 40–90 min). A further 2.8 hours passed, on average, before control cells with an unmanipulated free X chromosome entered anaphase.

How might mechanical force regulate the cell-cycle checkpoint? The essential link has just been discovered: spindle mechanics affect kinetochore chemistry⁷. Chromosome attachment to the spindle is followed by the dephosphorylation of certain kinetochore proteins⁷. Whether it is tension or some other feature of attachment that alters kinetochore chemistry remains to be seen, but the principle of mechanochemical linkage is established. Following McIntosh³, we propose that tension affects kinetochore chemistry, which in turn signals the checkpoint that all is well and sets in train the exit from mitosis. Other critical events probably follow the signal from the kinetochore, judging

from the long time lag between the application of force and the response, namely entry into anaphase. After these additional unknown processes are completed, the already known chemical events that herald the completion of mitosis follow, including cyclin degradation, kinase inactivation and protein dephosphorylation^{8–10}. These events are tied to the accurate distribution of chromosomes in cell division by an elegant use of mitotic forces not only to move chromosomes, but also to detect errors. □

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Interaction with RAP74 subunit of TFIIF is required for transcriptional activation by serum response factor

V ronique Joliot*, Mark Demma & Ron Prywes

Department of Biological Sciences, Columbia University, New York, New York 10027, USA

A FEW general transcription factors, in particular TFIID and TFIIB, have been found to bind transcriptional activators¹. Here we show that the general transcription factor TFIIF is also a target for a transcriptional activator, namely serum response factor (SRF), which binds to the *c-fos* promoter. Using a yeast interaction assay, we find that SRF binds the RAP74 subunit of TFIIF and that SRF's transcriptional activation domain is the region involved in this binding. Further, RAP74's central charged cluster domain is required for binding to SRF's activation domain. Deletion of this domain impairs RAP74's ability to support SRF-activated transcription *in vitro* but has little effect on the protein's basal transcription activity or its ability to support SP1-activated transcription. The correlation of SRF–RAP74 binding with transcriptional activation suggests that RAP74 is a critical target for SRF-activated transcription.

We previously found that transcriptional activation by SRF *in vitro* is particularly sensitive to levels of TFIIF and, using gel mobility shift assays, that SRF could bind the RAP74 subunit of TFIIF². To investigate this SRF–RAP74 binding we have used a yeast interaction assay in which SRF binds to its cognate site 5' to a β -galactosidase reporter gene but does not activate expression³. Proteins fused to VP16's transcriptional activation domain were then tested for interaction with SRF by their ability to activate the reporter gene's expression (Fig. 1b). VP16's activation domain was fused C-terminal to amino acid 437 of RAP74 (Fig. 2a) and we found that the RAP74–VP16 fusion protein could interact with SRF (Fig. 1b). As a positive control we used SAP1–VP16 (ref. 3), a SRF complexing protein, which caused activation of the reporter gene when expressed with SRF. Nega-

tive controls of RAP30–VP16 and VP16 or RAP74 alone did not activate. We also found that RAP74–VP16 did not activate transcription when expressed without SRF (data not shown).

SRF's transcriptional activation domain has been mapped to its carboxy terminus (amino acids 412–508)^{4–8}. We made mutations in SRF to determine whether binding to RAP74 correlates with the ability to activate transcription (Fig. 1a, c). Only constructs containing the C terminus were able to bind RAP74–VP16, whereas deletion of SRF's C terminus in SRF (1–412) abolished binding. We confirmed that all the SRF mutants were expressed in yeast by showing that they could still bind to SAP1–VP16, which binds to SRF through its DNA-binding domain^{9,10} (Fig. 1c). We found that SRF's transcriptional activation domain (amino acids 412–508) was sufficient to interact with RAP74 by using a yeast two-hybrid system with a GAL4-site reporter gene, GAL4's DNA-binding domain fused to RAP74 (GAL4–RAP74), and fragments of SRF fused to GAL4's transcriptional activation domain (GAD–SRF) (Fig. 1d). Deletion into the activation domain abolished the interaction (GAD–SRF[433–508]). This deletion also abolished transcriptional activation by GAL4–SRF constructs in NIH3T3 cells⁵.

We previously found using a gel mobility shift assay that SRF's core DNA-binding domain could also bind RAP74 (ref. 2). We investigated why this domain did not interact with RAP74–VP16 in the yeast interaction assay. One possibility was that RAP74's C terminus, which is deleted in RAP74–VP16, is required for binding to SRF's DNA-binding domain. We therefore tested for the interaction of SRF and full-length RAP74 fused to GAL4's transcriptional activation domain (GAD–RAP74; Fig. 2a, b). This experiment showed that the C terminus of RAP74 (amino acids 437–517) is necessary and sufficient for binding to SRF's DNA-binding domain. This binding, however, was weaker than the binding of GAD–RAP74 to full-length SRF, which was not affected by deletion of the C terminus. As described below, RAP74's C-terminal region is not required for transcriptional activation by SRF *in vitro*. Similarly, SRF's DNA-binding domain is not sufficient for activation of gene expression *in vivo*^{6,11} and only poorly activates *in vitro* (unpublished results). Therefore, the interaction of this domain with RAP74 does not appear to be required for transcriptional activation, but it may stabilize the binding of SRF to another region of RAP74.

We made mutations in RAP74–VP16 to determine other regions required for SRF binding. Binding of RAP74 to TFIIF's

* Present address: Institut Curie, Centre Universitaire Paris XI, 91405 Orsay cedex, France.

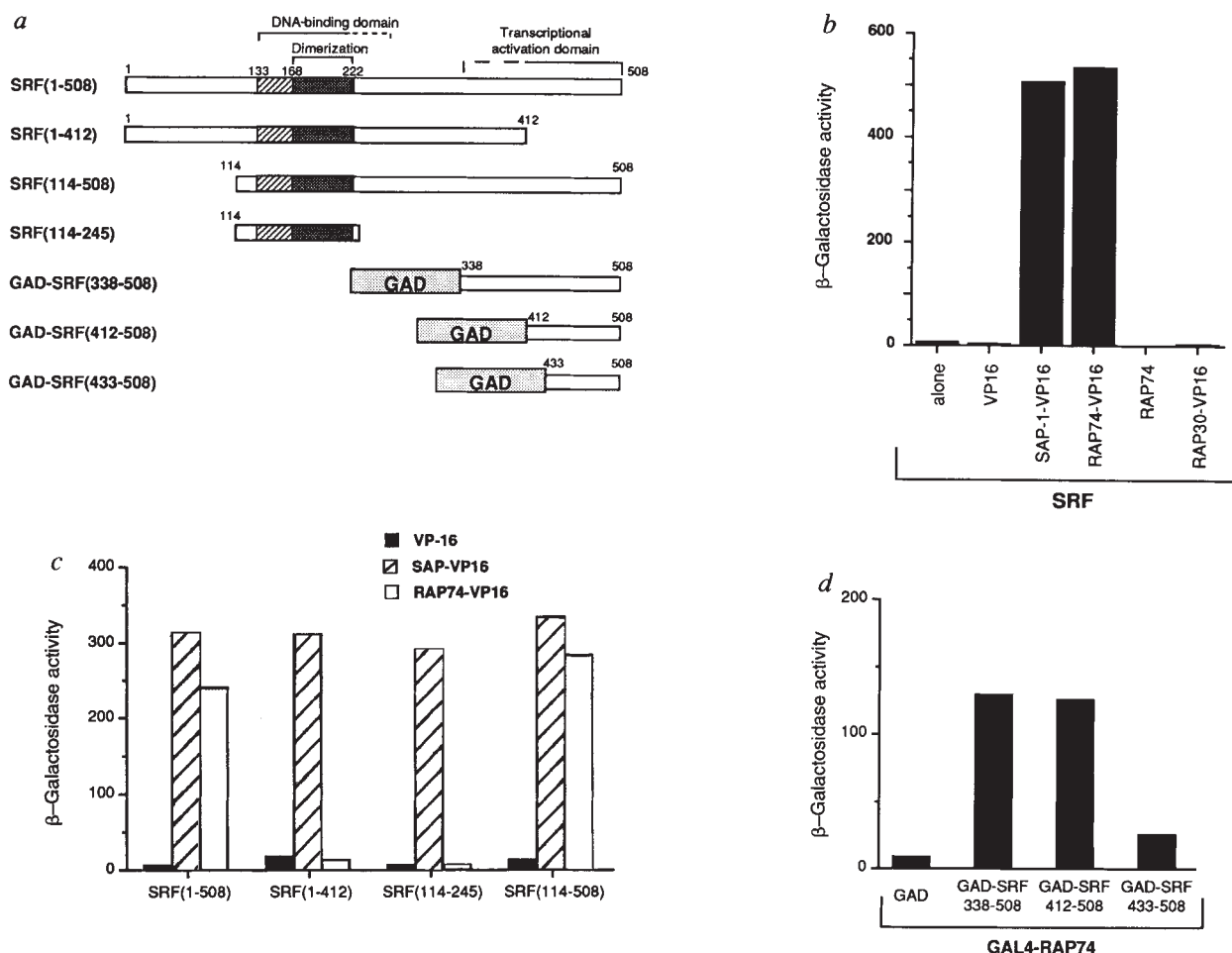


FIG. 1 Binding of SRF to TFIIF in a yeast interaction assay. **a**, Diagram of SRF and deletion mutants. The SRF expression vector, pSD0.7 (ref. 3), was used to generate the deletion mutants containing the indicated regions of SRF. In addition, fragments of SRF were fused to GAL4's transcriptional activation domain (GAD) in pGAD424 (Clontech) (details of constructions provided upon request). **b**, Full-length SRF was expressed alone or with the indicated proteins in *Saccharomyces cerevisiae* strain S8362L and β -galactosidase activity was measured as described³. This strain contains a lacZ reporter gene with an SRF binding site upstream of a *cyc1* promoter³. Amino acids 1–437 of RAP74 were used in the RAP74–VP16 and RAP74 constructs. This region of RAP74 was cloned into the VP16 expression construct pSD10³

N terminal to and in frame with the VP16 transcriptional activation domain. **c**, SRF's transcriptional activation domain is required for interaction with RAP74–VP16. The indicated SRF deletion mutants were expressed with VP16, SAP1–VP16 or RAP74–VP16 and β -galactosidase activity was measured. **d**, SRF's transcriptional activation domain is sufficient for binding to RAP74. GAL4's DNA-binding domain in pGBT9 (Clontech) was fused N-terminal to full-length RAP74 (GAL4–RAP74). GAL4's transcriptional activation domain from pGAD424 (Clontech) was fused N-terminal to the indicated fragments of SRF. The constructs were expressed together in yeast strain SFY526 (Clontech) containing a GAL4-site–lacZ reporter gene and the β -galactosidase activity measured.

second subunit, RAP30, has been mapped to amino acids 62–171 (ref. 12) and there is a central domain containing clusters of charged amino acids^{13,14} (Fig. 2a). Deletions in RAP74–VP16 showed that the central charged cluster domain was required for SRF binding (Fig. 2a, c). In particular, a RAP74 variant deleted for these sequences (Δ 206–291) was not able to bind SRF. We confirmed that this mutant was expressed stably in yeast by showing that it could activate reporter gene expression in conjunction with a GAL4–RAP30 fusion protein (Fig. 2d).

To determine whether the SRF-binding domains of RAP74 are required for SRF-activated transcription, we made a series of RAP74 deletion mutants in *Escherichia coli* and tested them for transcriptional activity *in vitro*. All of the mutants tested retained the RAP30 binding domain and basal transcription activity (Fig. 3a). For the basal transcription assay, purified general transcription factors were used, including recombinant TATA-binding protein (TBP) rather than HeLa cell TFIID, because TBP is sufficient for basal transcription and does not contain contaminating TFIIF.

We next tested for SRF-activated transcription using a partially purified HeLa cell TFIID fraction in addition to the other general transcription factors. The TFIID fraction was depleted of residual TFIIF contamination using an anti-RAP30-antibody affinity column. Although it was difficult to remove TFIIF from our transcription factor fractions completely, we were able to use them to demonstrate a clear dependence upon recombinant RAP74 for SRF-activated transcription (Fig. 3b, lanes 1–4). C-terminal-deletion mutants of RAP74 showed that amino acids 1–356 were fully functional for SRF-activation, deletion to amino acid 296 slightly reduced it and deletion to amino acid 206 nearly abolished activation (Fig. 3b). An internal deletion of amino acids 206–291 in full-length RAP74 (RAP74 Δ 206–291) did not support SRF-activated transcription effectively either (Fig. 3c), consistent with the requirement of this region for SRF binding. This mutant, however, was active for SP1-activated transcription (Fig. 3d). In Fig. 3c and d we used a cruder fraction of TFIID that had been depleted of TFIIF with anti-RAP30 serum. This fraction of TFIID was more effectively

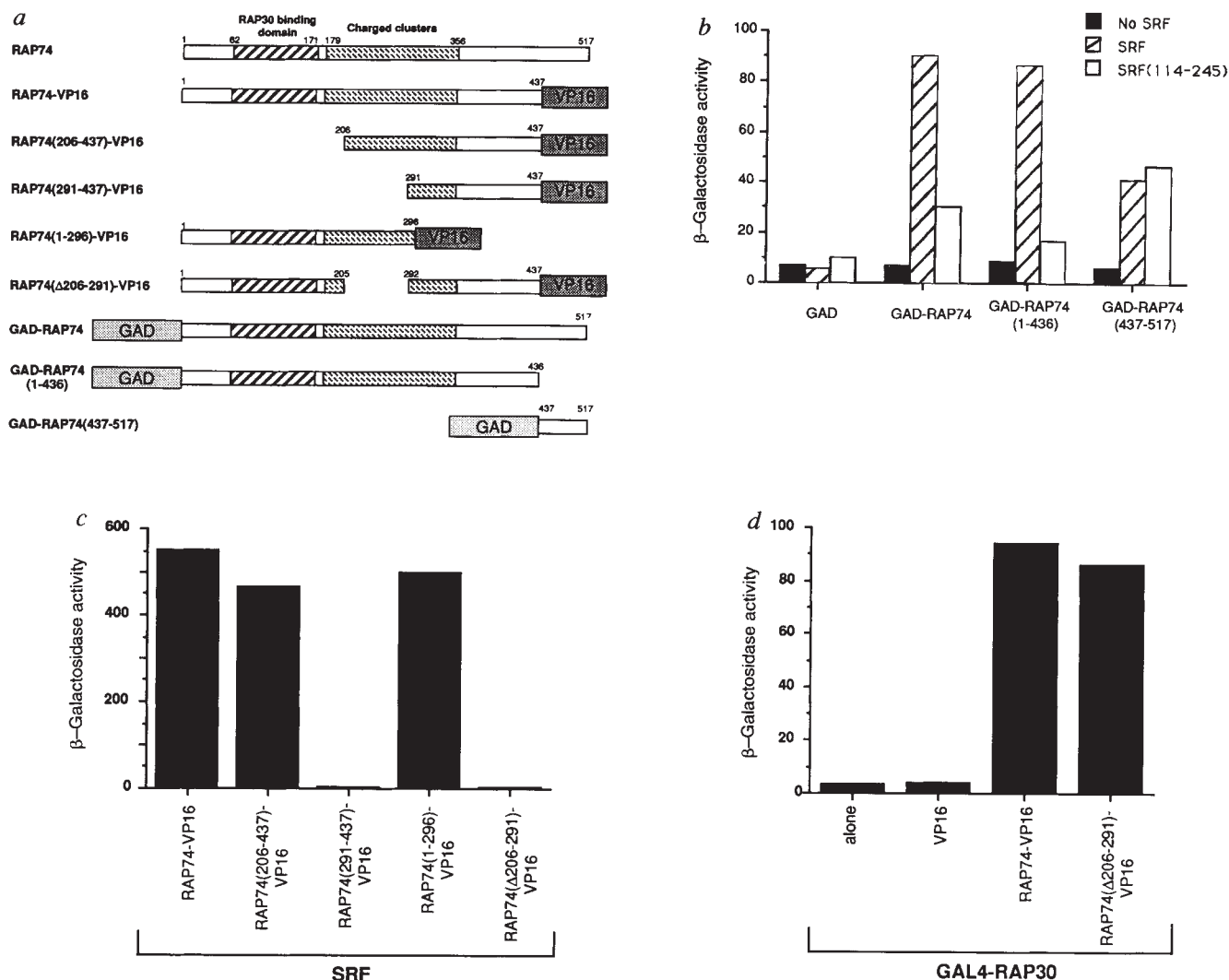


FIG. 2 Identification of RAP74's SRF-binding domain. **a**, Diagram of RAP74 and fusion proteins. The indicated mutants were expressed as fusion proteins with either VP16 or GAL4's transcriptional activation domain as indicated (VP16 or GAD). The RAP74-VP16 construct was as described in Fig. 1b. GAL4's transcriptional activation domain (GAD) from pGAD424 (Clontech) was fused N-terminal to full length RAP74 in GAD-RAP74. **b**, Binding of RAP74's C-terminal region to SRF's DNA binding domain. The indicated GAD-RAP74 constructs and an SRE-lacZ reporter gene were expressed alone, with full length SRF or with

SRF(114-245) as in Fig. 1b. **c**, SRF was expressed with the indicated RAP74-VP16 mutants and β -galactosidase activity was measured. **d**, The RAP74 deletion mutant was expressed and retained the ability to interact with RAP30. RAP30 sequences were cloned into pMA424 (ref. 22) to create a GAL4-RAP30 fusion protein. This plasmid was transformed alone or with the indicated constructs into yeast strain GGY1::171 (ref. 22) which has a GAL1-lacZ reporter gene and β -galactosidase activity was measured.

depleted of RAP74 and was more active in supporting SRF-activated transcription upon addition of RAP74, perhaps because it contains more cofactors for SRF-activated transcription. RAP74 did not consistently increase basal transcription (Fig. 3b and c), possibly because our crude TFIID fractions inhibit basal transcription. The smaller amount of wild-type RAP74 (20 ng; Fig. 3c) did not support SRF activation well either, consistent with our previous finding that there is a critical level of RAP74 required for SRF-activated transcription². There was a small amount of SRF activation with RAP74(Δ 206-291) which may be due to its binding to SRF's DNA-binding domain or to the interaction of SRF with other transcription components. The slight increase in activation with 80 ng of mutant was not reproducible. In addition, there was some SP1 activation in the absence of RAP74, presumably due to RAP74 contamination (Fig. 3d, lanes 1 and 2). This is consistent with our previous finding that less TFIIF is required for activation of SP1 than

for SRF². SP1 activation was slightly reduced with 80 ng RAP74(Δ 206-291) but, expression without SP1 was also less, so strong SP1 introduction was still observed (Fig. 3d, lanes 13 and 14).

In contrast to our results, Yonaha *et al.*¹² found that C-terminal deletion of RAP74 after amino acid 356 abolishes basal transcription. However, they may have been analysing activated transcription, because they used an adenovirus major late promoter that includes upstream activation sites and TFIIF-depleted nuclear extracts that may contain specific activators such as USF.

We have mapped the regions of SRF and RAP74 required for their mutual binding and have found that there is a good correlation between binding of RAP74 to SRF's transcriptional activation domain and RAP74's ability to function in SRF-activated transcription. These results suggest that SRF binding to RAP74 is important for SRF-activated transcription. TFIIF can

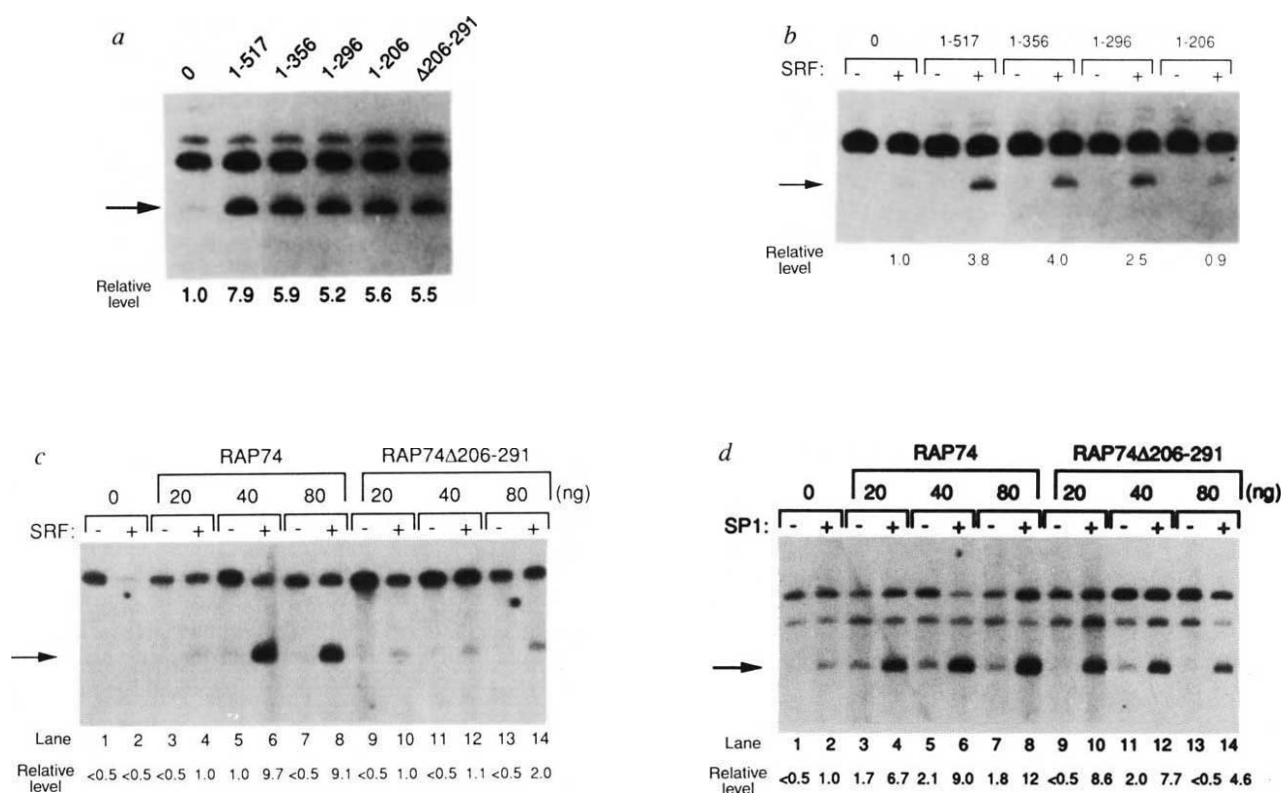


FIG. 3 RAP74 mutants defective for SRF-activated transcription *in vitro*. **a**, Basal transcription. The indicated RAP74 proteins (50 ng) were assayed for basal transcription activity. Accurately initiated transcripts (indicated by arrow) were detected by S1 nuclease analysis². The upper bands were from undigested probe or nonspecifically initiated transcripts. **b**, SRF-activated transcription. The *in vitro* transcription reactions were performed with or without SRF and the indicated RAP74 proteins (50 ng). The relative levels of the transcripts were quantified for SRF-activated lanes only as there was too little material in the other lanes for accurate quantitation. **c**, SRF-activated transcription. **d**, SP1-activated transcription. In **c** and **d**, increasing amounts of wild-type or mutant RAP74 were added as indicated. For **d**, the reactions were performed with or without SP1 with a template containing SP1-binding sites.

METHODS. *In vitro* transcription reactions and analysis were essentially as described². The template plasmids pFC53XGL (**a-c**) and pSVFC53.2

(**d**) contain either an SRF- or SP1-binding site, respectively, upstream of -53 of a minimal *c-fos* promoter². The general transcription factors used included recombinant TFIIB, TFIIE and RAP30, as well as partially purified RNA polymerase II, TFIIA and TFIIF². In addition, we used: in **a** recombinant TATA-binding protein (TBP); in **b**, TFIID purified from HeLa cells through phosphocellulose and DEAE-5PW columns; and in **c** and **d**, TFIID purified through a phosphocellulose column². The TFIID fractions were immunodepleted with anti-RAP30 serum as described². RAP74 was expressed with a poly-histidine tag using pET23d/RAP74 (ref. 23) and purified by nickel affinity chromatography²³. The RAP74 deletion mutants were derived from pET23d/RAP74. SP1 was purified from HeLa cell nuclear extracts by wheat germ agglutinin affinity chromatography. Expression was quantified using a phosphorimager (Molecular Dynamics). Experiments are each representative of at least three independent experiments.

bind RNA polymerase II (pol II) through its RAP30 subunit¹⁵ and in yeast is part of a large holo-pol II complex functional for activated transcription^{16,17}. Thus, SRF could promote recruitment of pol II to the promoter or change the conformation of the initiation complex. TFIIF also affects promoter

escape and transcriptional elongation^{18,21}, so SRF could potentially affect elongation steps. Further investigation will be required to determine whether other transcriptional activators bind RAP74 and how binding to RAP74 causes an increase in transcription. □

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Crystal structure of the GreA transcript cleavage factor from *Escherichia coli*

Charles E. Stebbins^{*†}, Sergei Borukhov[‡],
Marianna Orlova[‡], Andrey Polyakov^{*},
Alex Goldfarb[‡] & Seth A. Darst^{*}

^{*} Rockefeller University, 1230 York Avenue, New York, New York 10021, USA

[‡] Public Health Research Institute, 455 1st Avenue, New York, New York 10016, USA

[†] Present address: Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10021, USA

TRANSCRIPTION elongation factors stimulate the activity of DNA-dependent RNA polymerases by increasing the overall elongation rate and the completion of RNA chains. One group of such factors, which includes *Escherichia coli* GreA, GreB and eukaryotic SII (TFIIS), acts by inducing hydrolytic cleavage of the transcript within the RNA polymerase, followed by release of the 3'-terminal fragment¹⁻⁵. Here we report the crystal structure of GreA at 2.2 Å

resolution. The structure contains an amino-terminal domain consisting of an antiparallel α -helical coiled-coil dimer which extends into solution, reminiscent of the coiled coil in seryl-tRNA synthetases⁶. A site near the tip of the coiled-coil 'finger' plays a direct role in the transcript cleavage reaction by contacting the 3'-end of the transcript. The structure exhibits an unusual asymmetric charge distribution which indicates the manner in which GreA interacts with the RNA polymerase elongation complex.

The structure of the 158-residue protein GreA (Table 1 and Fig. 1a) is made up of two domains (Fig. 1b), each containing about half the residues. The overall shape is much like a capital 'L'. The long stem is about 63 Å long, and the short stem is about 35 Å. In the perpendicular direction the molecule is only about 15 Å thick.

The N-terminal domain (the long stem of the L) consists primarily of an antiparallel α -helical coiled-coil dimer (helices $\alpha 1a$, $\alpha 1b$ and $\alpha 2$ in Fig. 1b) that juts out about 45 Å into solution. Helix $\alpha 1$ has a bulge and a proline-induced kink (Fig. 1b), similar to the kink-bulge found in the heat-shock transcription factor DNA-binding domain⁷, leading to an unusual coiled-coil structure (Fig. 2). The characteristic heptad repeat, (abcdefg)_n, with hydrophobic residues at the a and d positions, is generally maintained throughout helix $\alpha 1$ from residues 8 to 23 (Fig. 2b). However, at Val 24, which is the residue 'bulged-out' of the helix, a one-amino-acid frameshift is required to maintain the heptad-

FIG. 1 a, Stereo image of GreA electron density at 2.2 Å resolution, generated using the program O¹⁵, showing a portion of the boundary between the N- and C-terminal domains. Shown in blue is the result of a $(2|F_{\text{observed}}| - |F_{\text{calculated}}|)$ difference Fourier synthesis, contoured at 1σ . Superimposed is the refined structure of the protein illustrated as atomic stick figures, using colour coding for atom type (C, yellow; O, red; N, blue; S, green), and water molecules, shown as white crosses. The larger cyan cross denotes the position of a well localized water molecule with a refined temperature factor of 10 Å^2 . Shown in orange is an $(|F_{\text{observed}}| - |F_{\text{calculated}}|)$ omit difference electron density map, contoured at 4σ , for the 6 water molecules participating in the hydrogen-bond network that bridges the N- and C-terminal domains of the protein. b, Stereodrawing of a Richardson-type²⁰ representation of the three-dimensional structure, generated using the program MOLSCRIPT (P. Kraulis). Residues were assigned to secondary structural elements according to criteria defined in ref. 21. The structure consists of an N-terminal, mostly coiled-coil, domain (made up of $\beta 1$, $\alpha 1a$, $\alpha 1b$, L, 3/10-1, $\alpha 2$, $\beta 2$ and 3/10-2), and a C-terminal, mostly β -sheet, domain (made up of $\beta 3$, $\beta 4$, L3, 3/10-3, $\beta 5$, $\alpha 3$, $\beta 6$ and $\beta 7$), connected by loop L2. The 24° kink between helices $\alpha 1a$ and $\alpha 1b$ is clear in this view.

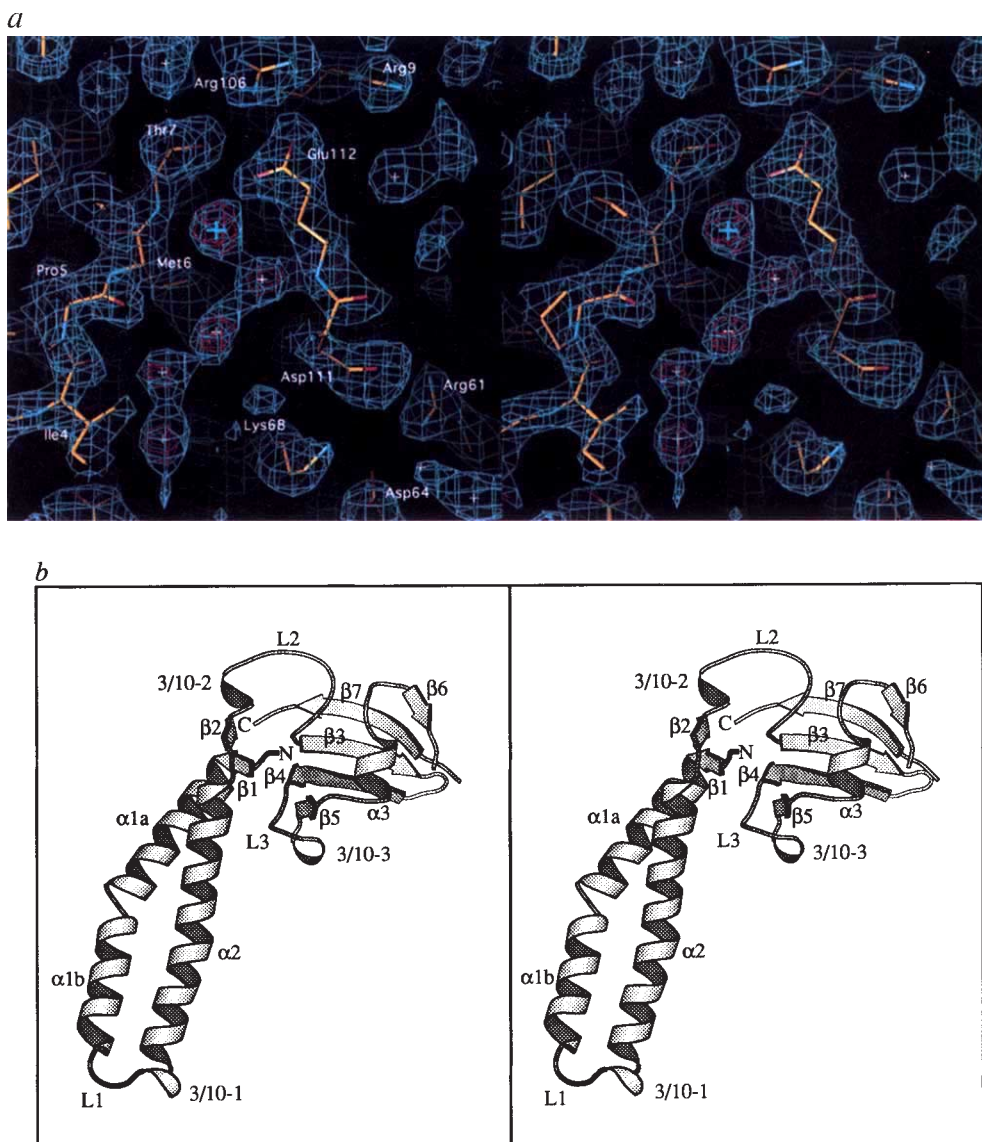


TABLE 1 Summary of crystallographic analysis

	Native	Se-Met	MMC1	MMC1	MMC1	MMC2	Se-Met/MMC
Diffraction data							
Wavelength (Å)	CuK α	CuK α	1.0091	1.0055	0.9919	0.9919	CuK α
Resolution (Å)	2.2	2.0	2.3	2.3	2.3	2.5	2.5
Reflections (observed/unique)	30,813/8,079	115,852/11,984	70,347/5,736	76,930/5,092	75,793/5,752	23,975/3,583	68,314/6,103
Completeness (%)	85.5	97.3	69.5	68.6	69.6	54.8	92.7
$I/\sigma(I)$	13.1	27.0	24.6	25.4	24.7	17.9	27.8
R_{sym} (%)	7.2	4.6	5.0	5.4	5.4	6.3	6.3
Phasing statistics (15–2.5 Å)							
R_{iso} (%)		20.7	14.5	14.9	15.3	17.9	21.3
Number of sites		2	1	1	1	1	2 (Se)/1 (Hg)
Phasing power		0.31	0.95	1.05	1.08	0.91	1.06
Mean overall figure of merit	0.70						
Refinement (6–2.2 Å)							
Resolution (Å)	(6–2.2)						
Reflections ($ F > \sigma F $)	8,040						
Total number of atoms in model	1,288						
Total number of solvent molecules	99						
R -factor (%)	20.9						
r.m.s. bond length (Å)	0.018						
r.m.s. bond angle (degrees)	2.134						

GreA purification and crystallization was as described previously¹². Se-Met GreA was produced from *E. coli* ER 1648 cells (New England Biolabs) transformed with pDNL278 (a gift from R. Landick) as in ref. 13. Purification and crystallization from microseeds of GreA was as described¹². Data collection was from cryoprotected crystals held at -180°C ¹². Data at the CuK α wavelength were collected on a Rigaku RAXIS-II imaging plate area detector. Methyl-mercury chloride (MMC) derivatives were formed by soaking crystals with 1 mM MMC for 24 h. MAD data were collected from two MMC-derivatized crystals (MMC1 and MMC2) at the HHMI beamline X4A (NSLS, Brookhaven, NY) using Fuji imaging plates. The wavelengths correspond to the inflection (1.0091 Å), the peak (1.0055 Å) and a remote value (0.9919 Å) of the X-ray absorption spectrum of the derivatized crystal. Data were processed using DENZO and SCALEPACK (Z. Otwinowski). Phasing was treated as an MIR problem with anomalous scattering for the MMC derivatives. Data sets collected at different wavelengths were treated as separate 'derivatives' using MLPHARE (Z. Otwinowski). The 2.5 Å MIR map revealed clear delineation between protein and solvent. A dramatically improved map was generated using SQUASH¹⁴, into which a polyaniline model of about 50% of the residues was built using O¹⁵. The map was improved by cycles of refinement using X-PLOR¹⁶, phase combination using SIGMAA¹⁷, and model building. Residues 1 and 142–147 do not have interpretable density and are not included in the model. Only 1 backbone torsion angle combination lies in an unfavourable region of the Ramachandran plot; 86% lie in the most favoured regions¹⁸. Coordinates will be submitted to the Brookhaven Protein Data Bank¹⁹. $R_{\text{sym}} = \sum |I - \langle I \rangle| / \sum I$, where I = observed intensity, $\langle I \rangle$ = average intensity from multiple observations of symmetry related reflections. $R_{\text{iso}} = \sum ||F_{\text{PH}}| - |F_{\text{P}}|| / \sum |F_{\text{P}}|$, where $|F_{\text{P}}|$ = protein structure factor amplitude, $|F_{\text{PH}}|$ = heavy-atom derivative structure factor amplitude. Phasing power = root mean square ($|F_{\text{H}}|/E$), where $|F_{\text{H}}|$ = heavy-atom structure factor amplitude and E = residual lack of closure. R.m.s. bond lengths and angles are the deviations from ideal values.

repeat pattern. The kink in helix $\alpha 1$, centred at Pro 27, is about 24° . The distal end of helix $\alpha 1b$ is linked to helix $\alpha 2$ by a loop (L1) and a 3-residue 3/10 helix (3/10-1). Just past the C-terminal end of helix $\alpha 2$, the backbone of Ala 72 is distorted from helical geometry but essentially occupies a 'd' position in the coiled-coil heptad repeat. Hydrophobic residues are conserved at this position in homologous Gre factors (Fig. 2b). The distortion contributes to the spacing apart of the helices, leaving a gap in the hydrophobic core that is filled by Met 6 (Fig. 2). Hydrophobic amino acids are also conserved at this position (Fig. 2b). The coiled-coil structure is stabilized by numerous interhelical hydrogen bonds, four interhelical salt bridges, and by a short, parallel β -sheet at one end ($\beta 1$ – $\beta 2$). The peptide chain continues from $\beta 2$ into another 3-residue 3/10 helix (3/10-2) and a 12-residue loop (L2) linking the N- and C-terminal domains.

The C-terminal domain is made up primarily of an α -helix ($\alpha 3$) cradled by a 5-strand β -sheet. The β -strand interactions are all antiparallel except for strands $\beta 4$ and $\beta 5$, which are parallel, and are connected by an 8-residue loop (L3) and a third, 3-residue 3/10 helix (3/10-3). The region of interaction between helix $\alpha 3$ and β -strands $\beta 3$, $\beta 4$ and $\beta 7$ defines the hydrophobic core of the domain, a portion of which is exposed at the solvent-accessible surface (Fig. 3a).

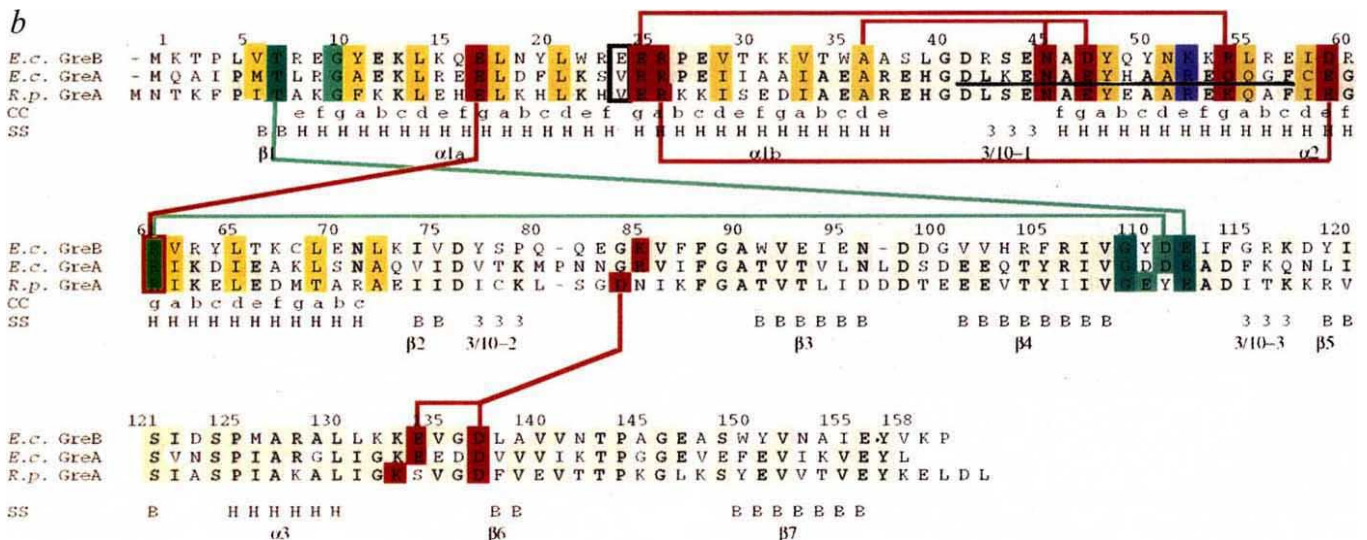
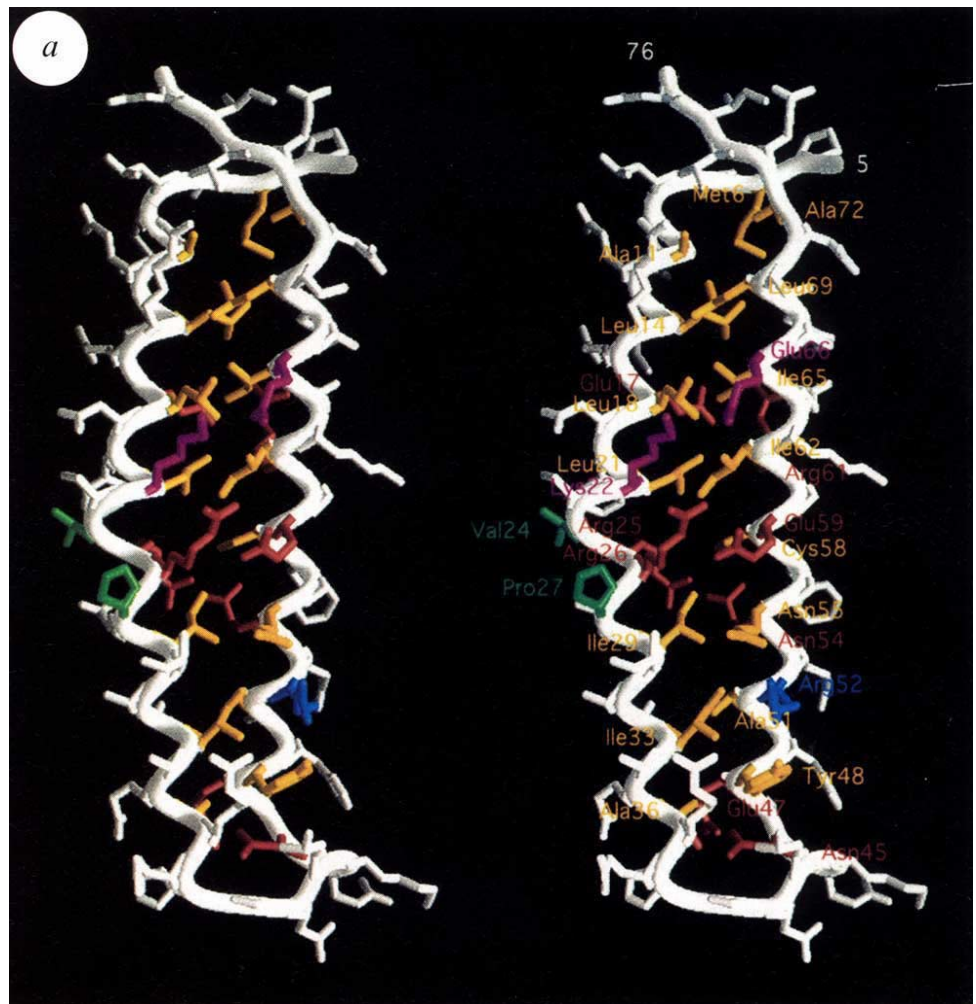
The boundary between the two domains is best described as a crevice penetrated by water molecules (Fig. 1a). Glu 112 is central to the structure of the domain interface, forming the only direct interdomain hydrogen bond (Thr 7(O γ)-Glu 112(O $\epsilon 1$)) and also interacting with a well localized, tetrahedrally coordinated water molecule (refined temperature factor of $10 \text{ \AA}^2$;

labelled with a large cyan cross in Fig. 1a) which is central to a group of six water molecules that form a hydrogen-bond network mediating interdomain contacts (Fig. 1a).

A striking asymmetry in the GreA structure is revealed by examining the charge-distribution around the water-accessible surface (Fig. 3a). One face of the molecule is strongly acidic (Fig. 3a, left), whereas the opposite face is neutral except for a small basic region, formed mainly by Arg 52, a conserved positively charged residue (Fig. 2b). The neutral face also includes the exposed hydrophobic patch.

Because GreA induces transcript cleavage near the RNA 3'-terminus within elongating complexes, we probed GreA-RNA interactions using transcription complexes of *E. coli* RNA polymerase containing nonamer transcripts with the photocrosslinkable nucleotide analogue 8-N₃-AMP (ref. 8) incorporated into the 3'-terminus⁹. On ultraviolet irradiation, crosslinking of the transcript to the β' subunit of RNA polymerase⁹ was observed (Fig. 3b, lane 3), as verified by 4% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and by crosslinking to RNA polymerase carrying His-tagged β' subunit (data not shown). In the presence of GreA, crosslinking of the transcript to GreA was also observed (Fig. 3b, lane 4). The yield of GreA-RNA photocrosslinking was roughly 7% (based on the initial amount of ternary complex), about 1/3 the efficiency of β' crosslinking. Two observations attest to the specificity of the crosslink. First, crosslinking of the RNA to BSA or lysozyme, added in large excess, was not observed. Second, crosslinking of the purified RNA to GreA (without RNA polymerase) was not observed.

FIG. 2 *a*, Stereodiagram of the *E. coli* GreA coiled-coil domain, generated using GRASP²². Shown as a white tube is the polypeptide backbone of residues 5–76. Side-chains are white except as follows: yellow, side chains of residues at *a* and *d* positions of the coiled-coil heptad repeat (except Arg 26), and also Ala 72 and Met 6; red, side chains participating in conserved, interhelical salt bridges and/or hydrogen bonds (Glu 17–Arg 61 and Arg 25–Asn 54 on the back, and Arg 26–Glu 59 on the front; also, Asn 45 forms hydrogen bonds with Glu 47 and the carbonyl oxygen of Ala 36). Magenta, an unconserved interhelical salt bridge (Lys 22–Glu 66); green, Val 24, the residue bulged out of the helix, and Pro 27, which induces the kink in helix α 1; blue, Arg 52, a conserved basic residue. *b*, Sequence alignment of *E. coli* GreB (*E. coli* GreB)⁴, *E. coli* GreA (*E. coli* GreA)²³, and *R. prowazekii* GreA (*R. p. GreA*)¹⁰, and schematic illustration of *E. coli* GreA structural features. The numbering above the sequences is with reference to *E. coli* GreA. The underlined sequence denotes the fragment containing the transcript 3'-terminus crosslink. Below are shown the following features of the *E. coli* GreA structure: CC, positions within the coiled-coil heptad repeat pattern; SS, secondary structure (B, β -strand; H, α -helix; 3, 3/10 helix); and labels for the secondary structural elements (Fig. 1*b*). The colour shadings represent the following: grey, amino-acid identities; yellow, *a* and *d* positions of the coiled-coil heptad repeat, with generally conserved hydrophobic residues (except Arg 26, which salt bridges with a negatively charged residue at position 59), and also conserved hydrophobic residues at position 6 and 72; black outline, helical-bulge which causes a frame-skip in the coiled-coil heptad repeat pattern; blue, conserved positively charged residues at position 52; light green, residues participating in interdomain interactions; red, interactions that stabilize the structure and show conserved complementarity.



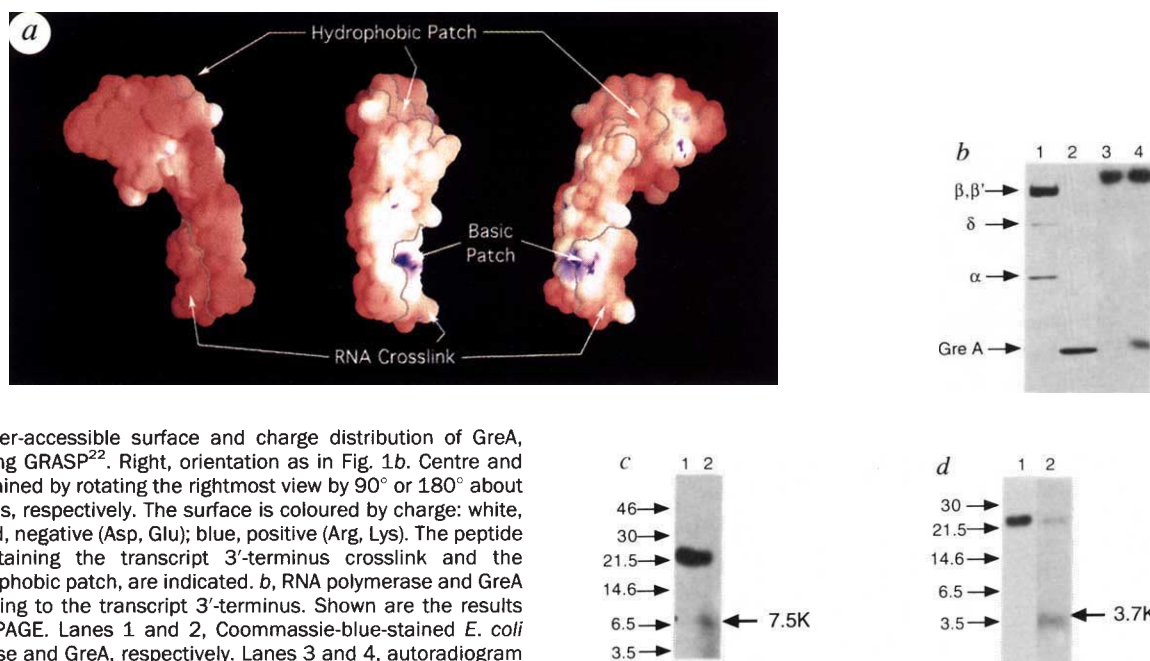


FIG. 3 a, Water-accessible surface and charge distribution of GreA, generated using GRASP²². Right, orientation as in Fig. 1b. Centre and left, views obtained by rotating the rightmost view by 90° or 180° about the vertical axis, respectively. The surface is coloured by charge: white, uncharged; red, negative (Asp, Glu); blue, positive (Arg, Lys). The peptide fragment containing the transcript 3'-terminus crosslink and the exposed hydrophobic patch, are indicated. b, RNA polymerase and GreA photocrosslinking to the transcript 3'-terminus. Shown are the results of 14% SDS-PAGE. Lanes 1 and 2, Coomassie-blue-stained *E. coli* RNA polymerase and GreA, respectively. Lanes 3 and 4, autoradiogram of UV-irradiated ternary complexes in the absence and presence of GreA, respectively. c, Mapping of the RNA crosslink site in GreA by cleavage at Cys residues²⁴. The autoradiogram of a Tris-tricine 16% SDS-PAGE²⁵ shows crosslinked GreA (lane 1) and products of the Cys-specific cleavage reaction (lane 2). Positions and masses (K) of markers are indicated on the left. Expected products from cleavage at the only Cys (position 58) of GreA are N- and C-terminal fragments of 6.5 and 11.1K, respectively. The autoradiogram revealed uncleaved GreA as well as a peptide with apparent molecular mass of about 7.5K, which we interpreted as the N-terminal fragment of GreA (Met 1–Phe 57) with additional mass from the crosslinked RNA. d, Mapping of the crosslink site by exhaustive enzymatic degradation at Asp residues²⁶ and thiol-specific chromatography. The autoradiogram of a Tris-tricine 16% SDS-PAGE shows crosslinked GreA (lane 1) and products of Asp-specific cleavage eluted from thiol-activated Sepharose (lane 2). The cleaved product contained 80% of the applied radioactivity. The autoradiogram revealed a band that, after taking into account mass from the RNA adduct, corresponds to the 2.7K, Cys-containing peptide fragment Asp 41–Lys 63.

METHODS. In b, purified complexes of *E. coli* RNA polymerase containing a hexamer transcript were prepared from the *rrnB* P1 promoter using CpA, ATP, and [α -³²P]CTP⁴. Addition of UTP, GTP and 8-N₃-ATP led to

formation of a complex containing a nonamer transcript with the analogue incorporated into the 3'-terminus. Reactions (total volume 25 μ l) in transcription buffer⁴ containing 7.5 pmol ternary complex, 1 mg ml⁻¹ BSA and 10 mM EDTA (to inhibit transcript cleavage), were supplemented with 5 μ g lysozyme (lane 3) or 3 μ g GreA (170 pmol, lane 4), and UV-irradiated⁹. The reaction was terminated by addition of dithiothreitol (DTT) to 50 mM, TCA precipitated, and resuspended in SDS loading buffer. c, Crosslinked GreA-RNA was isolated by 14% SDS-PAGE and electroelution⁹, supplemented with 5 μ g unlabelled GreA, and treated with 50 mM 2-nitro-5-thiocyanobenzoic acid in 8 M urea, 0.2 M Tris-HCl, pH 9.0, and 5 mM β -mercaptoethanol for 5 h at 37 °C²⁴. d, Purified GreA-RNA was supplemented with 5 μ g unlabelled GreA and digested overnight at 37 °C with 0.1 μ g endoproteinase AspN (Boehringer-Mannheim) in 100 μ l 50 mM Tris-HCl, pH 7.5, 0.05% SDS. The digest was precipitated with cold acetone and the pellet was redissolved in 40 μ l 50 mM Tris-HCl, pH 7.5, 8 M urea, 0.2% SDS. A 20 μ l suspension of thiol-activated Sepharose 4B (Pierce) in the above buffer (50% v/v) was added and incubated under argon for 30 min at 25 °C. The gel was centrifuged and washed twice with incubation buffer. The bound Cys-containing peptide was eluted with 40 μ l of the same buffer containing 50 mM DTT.

Proteolytic and chemical degradation were used to localize the site of RNA crosslinking to GreA within a 16-residue segment between Asp 41 and Phe 57 (Fig. 3c, d). This segment contains five residues that are conserved in GreA homologues (Fig. 2b), and also includes the conserved basic residue at position 52 and the basic patch on the protein surface (Fig. 3a). These results establish that the GreA coiled-coil finger plays a direct role in the transcript cleavage reaction by contacting the 3'-end of the transcript within the RNA polymerase catalytic site. The site of interaction with the 3'-end of the transcript is localized to a peptide segment within helix α 2, near the end of the coiled-coil.

In addition to *E. coli* GreB, a third GreA homologue has been identified from *Rickettsia prowazekii*¹⁰ (Fig. 2b), with 49% amino-acid identity to *E. coli* GreA. The biochemical activity of this protein has not been investigated. Examination of the sequence alignment reveals that key features of the *E. coli* GreA structure are conserved (Fig. 2b). These features include: (1) hydrophobic residues at the expected a and d positions of the coiled-coil domain and at positions 6 and 72; (2) all of the residues involved in interactions between the N- and C-terminal domains; (3) charge complementarity between residues that form interhelical salt bridges which stabilize the coiled-coil struc-

ture; (4) a positively charged residue at position 52 which contributes to the formation of the basic patch on the otherwise neutral face of the molecule within the fragment to which the RNA crosslink has been mapped (Fig. 3); and (5) the helical bulge at position 24, causing a skip in the coiled-coil heptad repeat pattern. On the basis of these observations, as well as the generally high amino-acid identities, it is highly likely that *E. coli* GreB and *R. prowazekii* GreA have very similar structures to *E. coli* GreA.

Although shorter by one heptad repeat, the GreA coiled-coil domain is most reminiscent of a similar feature of seryl-tRNA synthetase structures⁶. Interestingly, this coiled-coil arm also plays a role in RNA interactions, as revealed by the co-crystal structure of *Thermus thermophilus* seryl-tRNA synthetase complexed with tRNA^{Ser} (ref. 11).

The unusual charge distribution (Fig. 3a) suggests how GreA interacts with the elongation complex. The RNA polymerase itself is very acidic; the isoelectric point of the core enzyme, calculated from the total amino-acid composition, is 5.34. This does not take into account the negative charges of the phosphate groups of the DNA template and RNA transcript. A GreA molecule approaching an elongation complex would experience

electrostatic forces that would probably orientate the molecule with the strongly acidic side facing away from the polymerase. Accordingly, the neutral face, with its hydrophobic patch suggestive of a protein-binding surface, would be in a position to interact with the polymerase. This would presumably position the basic patch to contact the negatively charged transcript. Our crosslinking results established that a region including the basic patch, near the end of the coiled-coil, interacts directly with the 3'-end of the transcript during the cleavage reaction, as if the coiled-coil were a 'finger' poking into the RNA polymerase catalytic site. It is not possible at this point to propose a more detailed model of how GreA induces transcript cleavage, but the structure now allows testing of specific elements by biochemical and genetic means. □

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Supplementary Information is material relevant to Articles or Letters which cannot, for lack of space, be published in full, but which is available from *Nature* on request.

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Setting our sites on lasers

This week focuses on laser optics. A whole spectrum of accoutrements including a HeNe laser, an optical design tool system, a particle sizer and personal densitometer.

FOR shorter wavelengths and pulse duration, Coherent offers its 250-KHz, mode-locked, titanium-sapphire Mira/ regenerative **amplifier** (*Reader Service No. 100*). The RegA 9000/OPA system is designed to enhance applications in chemistry, physics and the biosciences. The advances in this system are said to be a result of the improved energy, stability and beam quality of the CW ion-pumped system. These characteristics are designed to allow the observation of the frequency-doubling of signal pulses from 480–700 nm in the visible range and 240–350 nm in the ultraviolet range. These pulse energies are in excess of 10 nJ. The visible OPA signal pulses also have been dispersion-compensated with a prism sequence to pulse widths as short as 70 femtoseconds, producing sufficient peak power to generate a white-light continuum. Further dispersion of this continuum spectrum is said to produce widely tunable, visible pulses with a duration of less than 30 femtoseconds.

A recent release from Melles Griot is the polarized, frequency-stabilized **HeNe laser system** intended for long-term frequency stability of ± 2 MHz over 8 h continuous use, and intensity stability of up to ± 0.1 per cent (*Reader Service No. 101*). The system has a simple turn-key locking feature for both frequency and intensity stabilization. The laser is suitable for applications such as interferometry, spectroscopy, metrology, velocimetry and microscopy. Packages for OEM users and optional wavelengths and powers are also available.

Wolfram Research has introduced **Optica**, an **optical system design and analysis tool** (*Reader Service No. 102*). Optic is a set of ready-to-use tools for the design of lasers and a wide range of holographic, spectroscopic, remote sensing, illumination and other optical systems. The system utilizes the mathematical and programming capabilities of Mathematica and offers a design environment that can be tailored to the needs of scientists, engineers and researchers. Its features include three-dimensional ray tracing, standard and customizable optical components, the ability to set parameters symbolically and publication-quality graphics. Optica provides the user with libraries of lenses, mirrors, prisms, gratings, pinholes, screens, optical fibres and other customizable components. It can define surfaces as spherical, parabolic, cylindrical and elliptical — as

well as others. Source code is included so that users can examine components or ray-tracing functions and modify or extend them for their own use.

Cambridge Consultants has developed a **laser micromachining system** for ultraviolet optically-assisted etching of ceramic infrared electro-optic chips, such as those used in high-resolution, room temperature, infrared imaging devices (*Reader Service No. 103*). The original system used one laser beam to cut microscopic grooves which form the individual detector elements that together comprise the image sensing array. The new system simultaneously generates five focused laser beams so the five grooves can be machined in parallel. This system uses an array of beam-splitting and conditioning elements to generate an angular array of beams that are focused by a custom ultraviolet objective.

■ Instruments

The **personal densitometer QC laser scanning system** from Molecular Dynamics offers quantitative analysis of gels, blots and films (*Reader Service No. 104*). This system includes performance validation features and documentation required



QC laser scanning system

for good manufacturing practice compliance in QC/QA, methods development and process development laboratories. Laser optics and 12-bit digital integration is intended to enable accurate densitometry with absorbance from 0 to 3.5 at a resolution of 50 μm . ImageQuaNT software, running under the Windows NT operating system, performs instrument control and data display and analysis.

Malvern has introduced the Mastersizer Micro and the Mastersizer S for **laser diffraction particle sizing** (*Reader Service No. 105*). Mastersizer S is intended for fast measurement of wet, dry, powdered or

aerosol samples in pharmaceutical research or quality control operations. This instrument can measure from 0.05 μm to 3,500 μm . There is security protection to limit data access and assist with good laboratory practice and with validating procedures. The Micromaster Micro utilizes a single lens arrangement designed to enable users to measure suspended particles from 0.3 μm to 300 μm .

■ Peripherals

The new DataSYS 940 from Gould Instruments is a **digital storage oscilloscope** that combines low-noise inputs with a bandwidth of 350 MHz to ensure signal fidelity (*Reader Service No. 106*). This instrument uses an application specific integrated circuit preamplifier designed for lower noise levels. The 350-MHz bandwidth is said to be guaranteed on all input sensitivity ranges from 5 V per division making the 940 model suited to applications such as digital system development and analog circuit noise debugging. The display modes include refresh, persistence, roll, x/y, with pre- or post-trigger and live zoom. For users working with complex equipment who require more than waveform acquisition and display, the instrument offers a choice of storage capabilities, wave-form analysis, automatic measurements and long-term archiving for later reference. Complete application solutions can be provided by adding a number of low-cost options which are said to integrate easily to tailor the instrument to the user's requirements.

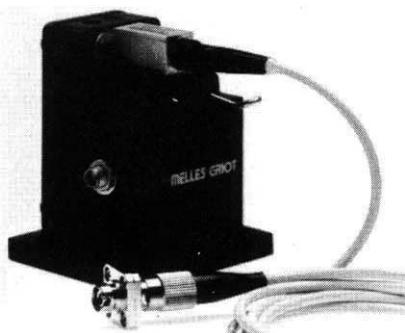
The Yokogawa TC100 series, available from Martron Instruments, is a new generation of **programmable timer-counters** with a general purpose interface bus — the series offers voltage-measuring capabilities in addition to frequency and period measurement (*Reader Service No. 107*). Martron offers two models: the TC110 which covers the frequency range from 1 mHz to 120 MHz; and the TC120 which uses a 1/128 prescaler to increase the frequency measuring range to 2 GHz. For period measurement, both instruments will measure from 20 ns up to 1,000 s. The nine-digit display can be configured to show two simultaneous three-digit indications of high- and low-peak voltage levels, so that it is not necessary to use a separate oscilloscope for this function. Reciprocal frequency counting is used to give eight-digit resolution in one second, and a single-shot resolution of 10 ns is provided by the multiphase clock system. The trigger level

is displayed digitally, and may be changed and confirmed on the display without interrupting frequency measurements. Trigger levels can be in 20-mV increments.

Miscellaneous

Also from Gould Instrument Systems is the KombiGraf Series single **compact oscilloscope** (*Reader Service No. 108*). This instrument has the integrated functions of a real-time analog oscilloscope, a digital storage oscilloscope and a chart recorder. Featuring a 20-MHz, real-time bandwidth and ten megasample-per-second sampling, the unit incorporates a direct-action control system that is said to make different functions easy to use and to allow the acquisition technique to be selected for a particular application. As an oscilloscope, KombiGraf is suited to the real-time viewing of repetitive signals and the capture, storage and display of transient signals. In the recorder mode, this instrument is said to offer the benefits of high bandwidth, high speed and low paper cost when compared to laboratory instruments such as ultraviolet oscillographic recorders. In both modes, the instrument allows long records of slowly changing phenomena to be made while maintaining the ability to view fast transients or glitches.

Melles Griot has announced the release of a **heat sink/mount** designed specifically for use with 14-pin dual in-line lasers (*Reader Service No. 109*). This heat sink/mount is designed to allow temporary



Melles Griot heat sink/mount.

or permanent mounting for these communications-style diode lasers during laboratory and field testing. Mounting the laser into the heat sink is said to be straightforward, as the diode laser simply plugs into the socket connector and is secured with thumbscrews. In addition to providing secure electrical contact with the mount, the screws ensure that there is good contact for proper heat sinking of the laser. The socket of the heat sink/mount is electrically connected to an eight-pin adapter for all connections once the diode laser is installed into the socket. All I/O connections are made from the back of the mount

which eliminates interference of cables and wiring with the fibre-coupled output.

Melles Griot now offers a series of **fibre pigtailed diode laser assemblies** which couple diode laser radiation into single- and multimode fibres (*Reader Service No. 110*). The assembly is available with both visible and infrared diode lasers, and is offered in a vast array of wavelengths and output power levels. The infrared and visible assemblies are stated to be capable of greater than 55 per cent and 30 per cent optical efficiency, respectively. A choice of diode lasers is available with nominal wavelengths of 635 nm to 830 nm and output power levels of 0.5 mW to 30 mW at typical operating currents. This series produces a circular beam, free of astigmatism and spherical aberration. The housing is precision manufactured stainless steel for enhanced durability and stability over temperature. The package size is less than 40 mm in length and each unit comes with a one-metre length of optical fibre. The standard product has a cleaved end and contains a flange that allows for mechanical positioning and heat sinking of the package. A variety of connectors are available as fibre terminations. This system is designed for use in applications where shock, vibration and temperature conditions are not optimal. Custom versions are available on request.

Catalogues

L.O.T.-Oriol has announced the publication of the new product **guide for light sources, monochromators and detection systems** (*Reader Service No. 111*). This 528 page book is designed to be a technical reference and product catalogue. There is information on nitrogen and tunable dye lasers, calibrated irradiance sources, single- and dual-beam scanning spectrophotometers, imaging monochromators/spectrographs with multiple grating turrets, cooled UV-IR detectors and charge coupled devices for spectroscopy and digital lock-ins.

Acton has released the new **eximer and ultraviolet laser optics and coatings catalogue** (*Reader Service No. 112*). This catalogue offers optics for eximer, ultraviolet and vacuum-ultraviolet lasers, including 172 nm, 193 nm (ArF), 222 nm (KrCl), 248 nm (KrF), 308 nm (XeCL) and 351-353 nm (XeF).

The new EG&C Ortec **radiation detection, measurement and analysis catalogue** contains tutorial information, applications advice and instrument selection charts for the research scientist (*Reader Service No. 113*). It is intended to cover a range of disciplines including: nuclear spectroscopy, multichannel scaling, mass spectroscopy, laser infrared

radar, fluorescence lifetime, single-photon counting, picosecond timing and gamma-ray or alpha-particle spectroscopy. EG&C Ortec manufactures radiation detection and associated electronic modules, in addition to instruments and systems for radiation detection, measurement and analysis. □

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